UNIVERSITY OF UDINE

RESEARCH DOCTORATE IN ECONOMICS, ECOLOGY AND PRESERVATION OF AGRICULTURAL AND LANDSCAPE-ENVIRONMENTAL SYSTEMS

(24TH CYCLE)

PHD DISSERTATION

METAL TOLERANCE AND ECOLOGICAL CHARACTERIZATION IN POPULATIONS OF A PSEUDOMETALLOPHYTE *BISCUTELLA LAEVIGATA* L.

DOCTORAL CANDIDATE: FILIP POŠĆIĆ

SUPERVISOR: PROF LUCA MARCHIOL

COORDINATOR OF THE DOCTORATE: PROF MARGHERITA CHANG TING FA

ACADEMIC YEAR 2010/2011

Dedication

This thesis is dedicated to my family and my fiancé Natalia. It is especially dedicated to my father who has inspired my curiosity for Nature.

Acknowledgements

The author wishes to express his gratitude to Prof Marchiol, Prof Schat and Prof Vischi, for their guidance and coordination regarding this research, and Dr Fellet for his assistance. Deepest gratitude are also due to the reviewers, Prof Gabbrielli and Prof Marmiroli. The author would also like to convey thanks to the Ministry and Faculty for providing the financial means and laboratory facilities. Special thanks also to all my friends.

Contents

1
General introduction

Filip Pošćić

1.1. Metal Contamination

Metal contamination in soils can occur naturally, such as through ore outcropping, or anthropogenically, from the time its ore is being mined to the time it becomes usable as a finished product or ingredient of a product. Potentially contaminated soils may occur at old landfill sites, old orchards that used insecticides containing arsenic as an active ingredient, fields that had past applications of waste water or municipal sludge, areas in or around mining waste piles and tailings, industrial areas where chemicals may have been dumped on the ground, or in areas downwind from industrial sites (USDA, 2000). Metal radionuclides contamination can also occur in modern nuclear era due to nuclear weapon tests and nuclear accidents.

Non-essential metals can be toxic at low concentration while essential metals can become toxic when present at high concentrations. Soils contaminated with non-essential metals can result toxic for some plant species due to incomplete ion selectivity of the trans-membrane carriers responsible for the uptake of essential elements. Toxic levels of metals in plants can interfere with metabolic pathways resulting in a homeostatic imbalance.

1.2. Metal tolerance

Metallophytes are a small group of higher plant species that have the ability to survive and reproduce on soils containing high concentrations of metals, the so called metalliferous soils. Metalliferous soils contain high concentrations of metals in forms that are toxic or inimical to other plants (Macnair and Baker, 1994). Mining sites represent a good ecological niche for metallophytes (Batty, 2005) and hyperaccumulators have been used to identify mineralized rocks and ores (Cannon, 1960).

The relation of metal tolerance and ecological range across different soils within and between plant species can be theoretically represented by Figure 1.1 from Pollard *et al.*, 2002. Each species is shown with a normally distributed variance of genotypes that encompasses all populations of the species in question. The lower x-axis is the bioavailable metal concentration in the natural soils in which these species grow. The upper x-axis indicates the physiological degree of metal tolerance of the genotypes. Note, that the threshold values above which soils are described as metalliferous and plants as metal tolerant do not exactly coincide, so there is a zone across which some metal-tolerant genotypes are found on nonmetalliferous soils. The majority of plants are

sensitive to metal toxicity so they are strict or obligate non-metallophytes (species A). Eumetallophytes or edaphic endemics are strict metallophytes, i.e. able to grow only in metalliferous soils (species D). On the contrary, pseudometallophytes occur in both metalliferous and non-metalliferous soils and therefore they are facultative metallophytes (species B and C). In species B, genes for metal tolerance may be present at low frequencies even in populations existing on non metalliferous soils (light shading in Figure 1.1). When plants colonize metalliferous soils (heavy shading in Figure 1.1), there is strong selection favouring the metal-tolerant genotypes, causing a rapid increase in the average tolerance of the local populations and leading to the establishment of metal-tolerant "races" or ecotypes (Antonovics *et al.*, 1971; Ernst, 1974). The species C represent a species typically found on metalliferous soils but their ecological range also

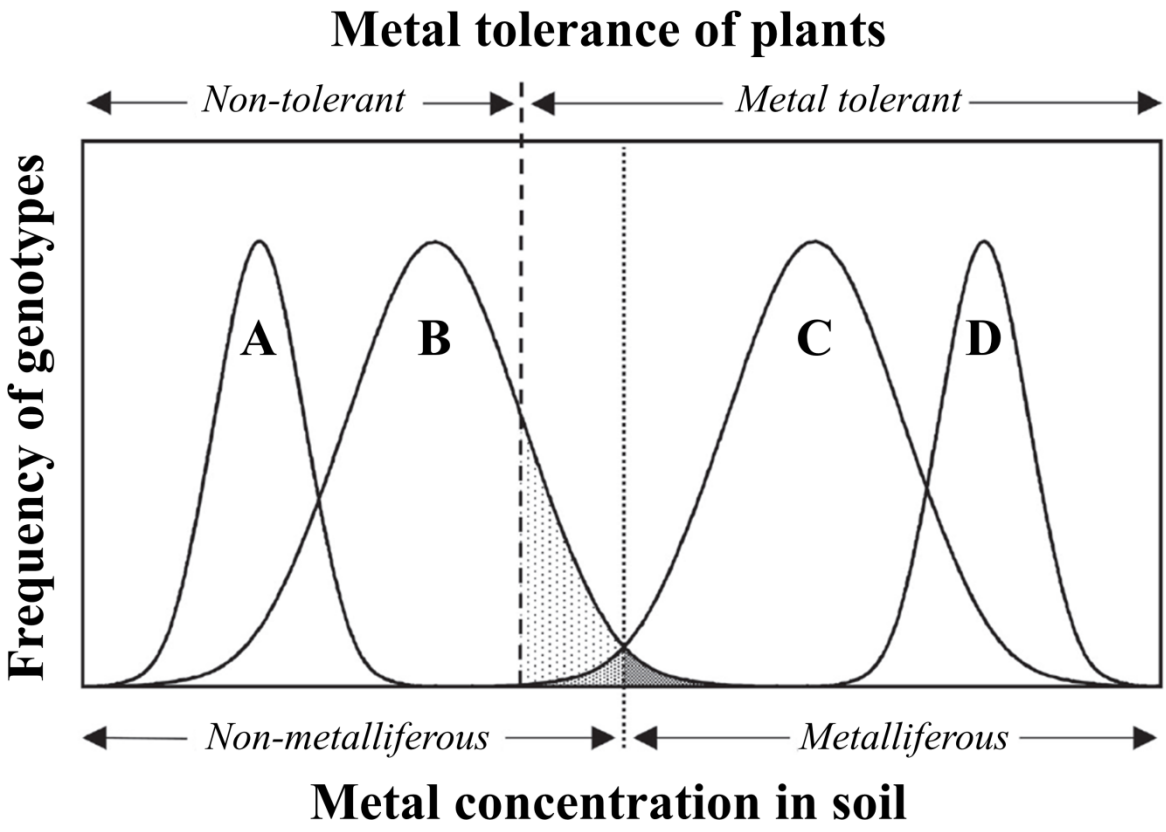

Figure 1.1: – Patterns of plant metal tolerance and frequency distribution of plant genotypes on metal-containing soils exemplified for four contrasting species (A, B, C, D). (A) strict or obligate non-metallophytes; (B) facultative metallophytes; (C) facultative metallophytes with species-wide tolerance; (D) strict or obligate metallophytes. The ecological ranges of the facultative metallophytes (B, C) are shown as being greater than those of the strict non-metallophyte (A) and strict metallophyte (D). Zones of light, intermediate, and heavy shading for the facultative metallophytes illustrate either the minority of genotypes within the species that are metal tolerant (B), or the minority within the species that occur on non-metalliferous soils (C). The x-axes are continua and the boundaries between tolerant/nontolerant and metalliferous/nonmetalliferous are to some extent arbitrary (originally from Pollard *et al.*, 2002).

includes populations occurring on non metalliferous substrates (illustrated by intermediate shading in Figure 1.1). Thus, in species B only a minority of the genotypes are metal tolerant, whereas in species C only a minority are non-metallophytes (Figure 1.1). An important feature of Figure 1.1 is that each of the four types of species depicted possesses some degree of intraspecific genetic variance in metal tolerance. Types A and D are shown with narrower variance than B or C, because of the more restricted range of soils that they inhabit, but even these species are likely to possess some variability in their sensitivity to soil metals.

A more restricted group of metallophytes named hyperaccumulators are capable of sequestering metals in their shoot tissues at remarkably high concentrations that would be toxic to most organisms. Hyperaccumulators are plants defined as capable to accumulate more than 100 mg kg^{-1} of Cd, 1,000 mg kg^{-1} of Ni, Co, Cr, Cu, Pb, Tl and 10,000 mg kg^{-1} of Zn and Mn in the dry matter of the plant shoots (Baker and Brooks, 1989; Krämer, 2010).

The physiological mechanisms of tolerance have been categorized as either exclusion, blocking the movement of metals at the soil/root or root/shoot interface, or accumulation, allowing uptake of metals into aerial parts and rendering them non-toxic through chemical binding or intracellular sequestration (Baker, 1981; Baker and Walker, 1990; Ernst *et al.*, 1992). In general, combinations of all these mechanisms do contribute to metal tolerance. Furthermore, in most plants, metals are predominantly accumulated in the roots. As a consequence, shoot/root ratios (the so called translocation factor, TF) of metal concentrations are generally substantially below unity in these plants. On the contrary metal concentrations TF are high in accumulators. Hyperaccumulators can be seen as a special and extreme case of the broader category of accumulators where metal concentrations are much higher in the leaves then in the roots, with metal concentrations TF abnormally high and above unity (Krämer, 2010).

Metal tolerance to different metals in the same species can rely either on the possession of combinations of different metal specific tolerance mechanisms, or on less specific mechanisms that pleiotropically confer tolerance to different metals (Schat and Vooijs, 1997). Metal hyperaccumulation involves strongly enhanced metal partitioning from the root into the shoot and very high levels of metal tolerance (Krämer, 2010).

The genetic and molecular basis of metal hyperaccumulation is given in a recent review from Krämer (2010). It is evident that the metal hyperaccumulation requires complex alterations in the plant metal homeostasis network and it can be due to

membrane transporters of metals, metal chelation and antioxidants in metal hypertolerance. Krämer (2010) reports there are some candidate genes identified for Zn and Cd hypertolerance as *Heavy Metal ATPase 4* (*HMA4*) and *vacuolar membrane* Zn/H^+ *antiporter* (*MTP1*). *HMA4* encodes a plasma membrane protein of the heavy metal pump family of P-type ATPases. *HMA4* and *MTP1* transcript levels are overexpressed in hyperaccumulators when compared to closely related non-accumulators. Moreover the Zinc-regulated transporter, Iron-regulated transporter-related protein family (ZIP) is also involved in metal hyperaccumulation but its individual components functions remain to be identified.

Regarding metal chelation (Krämer, 2010) histidine was shown to have a key role as a Ni chelator while Nicotianamine (NA) exhibits very high stability constants for the binding of all transition metal cations. Both, histidine and NA are present in higher concentrations in the hyperaccumulators when compared to closely related non-accumulators. There are present also proteins like yellow stripe-like membrane transporters (YSL) which are involved secondly to the hypertolerance. Organic acids (malate or citrate) are involved in the chelation of metals, too.

Finally after a metal intoxication the plant can response with antioxidants through glutathione (GSH) mediated reduction (Krämer, 2010). Glutathione can also act as a metal chelator and as a substrate for the biosynthesis of phytochelatins (PCs). PCs are low-molecular-weight, metal-chelating compounds known to be required for basic metal tolerance found in all plants. However, it seems that GSH, and therefore PCs, are not involved in hypertolerance.

It is then evident that metal tolerances appear to be determined by one or a few single major genes and influenced by a variable number of hypostatic minor genes referred to as 'modifiers' or 'enhancers' (Macnair, 1993; Schat *et al.*, 1996; Schat and ten Bookum, 1992; Smith and Macnair, 1998). Hypostatic modifiers are defined as genes of which the phenotypic expression is dependent on the presence of particular alleles at another locus.

Despite the large number of studies that have been carried out on common heavy metals and metalloid, there is a lack of knowledge according to Tl and Cs tolerance and hyperaccumulation.

1.3. The case of Thallium

Thallium is a heavy metal (IIIA group) with number 81 in the periodic table of

elements. There are two stable isotopes: 203-T1 (29.52% natural occurrence) and 205-T1 (70.48% natural occurrence). Thallium can occur in the zero-, mono- and trivalent state. The oxidative state +3 is named thallic while the +1 thallous which is more common, stable and less toxic. The atomic weight is 203 and the melting point 303.5°C. Thallium is moderately volatile as the element, as well as halogenide, oxide or nitrate. Inorganic Tl(I) compounds are more stable than Tl(III) analogues in aqueous solution at neutral pH. In fact compounds of Tl(III) thermally decompose to yield the respective T1(I) compounds, and the organic derivatives pyrolyze to Tl^0 or $T1_2O$ (Greenwood and Earnshaw, 1984; Galván-Arzate and Santamaría,1998). In contrast, covalent organothallium compounds are stable only in the trivalent form (Mulkey and Oehme, 1993).

Thallium was accidentally discovered by Sir William Crookes in 1861 by burning the dust from a sulfuric acid industrial plant. He observed a bright green spectral band that quickly disappeared. Crookes named the new element "Thallium" (after Greek θαλλός, thallos, meaning young shoot). In 1862, Claude-Auguste Lamy described the same spectral line and studied both the physical and chemical properties of this new element (Prick, 1979).

Thallium is present in the environment as a result of natural process and from man-made sources. Only a few areas contain a naturally very high-thallium concentration (Saha, 2005) as Alšar deposit which is a gold – arsenic – antimony – thallium deposit in southern Macedonia. Alšar deposit was mined extensively between 1880 and 1908. Antimony, arsenic and thallium were produced during that period. (up to 2% Tl in the soil) (Percival and Radtke, 1994). The few Tl(I) minerals existing contain about 16 – 60% of the metal and they are quite rare: Crookosite ($(Cu, Tl, Ag)_2Se$), Hutchinsonite ($(Tl, Ag)_2S{\cdot}Pbs{\cdot}2As_2S_3$), Lorandite ($Tl_2S\ AsS_3$), and Vrbaite ($Tl_2S_3(As,Sb)_2S_3$). Natural thallium concentrations in seawater and freshwater are estimated to be 0.03 ppb (Mulkey and Oehme, 1993). In soils not exposed to anthropogenic pollution, Kabata-Pendias (2001) reported a Tl concentration within the range 0.02 – 2.8 mg kg^{-1}.

Thallium toxicity to mammals is greater than Cd, Cu, Hg, Pb or Zn (Zitko, 1975). Similarly, Tl is considerably more toxic than Cd, Fe, Pb and Zn in plants (Allus *et al.*, 1988). Therefore, Tl is listed by USEPA as one of the 13 priority pollutants (Keith and Telliard, 1979). Tl(I) is particularly toxic in its Tl(I) compounds, such as sulphate (Tl_2SO_4), acetate (CH_3COOTl), and carbonate (Tl_2CO_3). The sulfide (Tl_2S) and iodide (TlI) are both poorly soluble and therefore, much less toxic (Moeschlin, 1980). Heim *et al.* (2002) report that Tl(I) toxicity to terrestrial invertebrates and plants is 100 times

higher than that of Cd. Similar results were obtained by Relph and Twiss (2002) where they assessed that in term of toxicity Tl(III)>Cd(II)>Tl(I).

Inside the living cell, stronger affinities towards S- and P- containing ligands lead to substitution of K at specific adsorption sites. This interferes with K-metabolism and, in many cases, with the formation of ATP by respiration (Sager, 1994).

The toxicity of Tl(I) can be effectively mitigated by K-ion at similar concentrations. Moreover, early studies have shown that Tl is a K analog and competitor. This antagonism has been shown in growth of *Aspergillus flavus* and in seed germination of *Plantago maritima* (Siegel and Siegel, 1975, 1976a). Potassium deficient *Cucumis sativus* cv Marketer seedlings resulted more sensitive to thallium then those receiving exogenous potassium (Siegel and Siegel, 1976b). Radić *et al.* (2009) have shown that Tl is responsible for DNA damage in *Vicia faba* seedlings by inducing oxidation stress.

Thallium can be readily taken up by plants because it is generally present in soil as thermodynamically stable Tl(I) and, as such, is an analogue of potassium. Moreover Tl(I) is likely the major species present in the different plant parts (Günther and Umland, 1989; Krasnodębska-Ostręga *et al.*, 2008).

Most of crops grown in non-contaminated soils had Tl contents comprised in the range 0.02-0.04 mg kg^{-1}. Values of 2 to 7 mg kg^{-1} are considered anomalous (Adriano, 2001). A great amount of studies found large differences among species of plants in Tl uptake and translocation (Tremel *et al.*, 1997; Anderson *et al.*, 1999; Scheckel *et al.*, 2004; Tangfu *et al.*, 2004; Wierzbicka *et al.*, 2004; Al-Najar *et al.*, 2005; Pavlíčková *et al.*, 2006; Madejón *et al.*, 2007).

Due to its low natural abundance and the increasing demand, Tl is the fourth most expensive metal, thus, recovery and reuse could be a profitable endeavour. Tl(I) is used as a catalyst in certain alloys, optical lenses, jewellery, low temperature thermometers, semiconductors, dyes and pigments and scintillation counters. Tl(I) has also been employed for clinical purposes as a epilator (Marmo *et al.*, 1987). In addition, Tl salts are still widely used as rodenticides and insecticides in some countries, despite the World Health Organization recommendation against its use in 1973.

1.4. The case of Caesium

Caesium is an alkali metal that exists in solution almost exclusively as the monovalent cation Cs(I) (Greenwood and Earnshaw, 1984). It is the most electropositive and active of

all metals; it oxidizes readily and forms strong bases. These and other properties, such as a specific gravity of lower than five, suggest that according to many definitions Cs is not a heavy metal, although it should be stressed that these definitions are mostly arbitrary and do not necessarily predict the behaviour of a metal in a biological or environmental context (Gadd, 1992).

Caesium was discovered by Robert Bunsen and Gustav Kirchhoff in 1860 by the newly developed method of flame spectroscopy. This new element was named "caesium" after the two prominent blue lines of its emission spectrum (from Latin caesius, meaning heavenly blue) (Burt, 1993). Caesium is used in small quantities in a variety of uses such as a high-density component in drilling mud used for petroleum exploration, component in television image devices, night-vision equipment and solar photovoltaic cells. In 1967, a specific frequency from the emission spectrum of ^{133}Cs was chosen to be used in the definition of the second by the International System of Units. Since then, Cs has been widely used in atomic clocks (Butterman *et al.*, 2005).

The stable ^{133}Cs isotope is the rarest of the alkali metals, although trace quantities of stable Cs do occur in most living organisms (Avery *et al.*, 1991; Davis, 1963; Komarov and Bennett, 1983). Concentrations of naturally occurring stable isotope ^{133}Cs vary considerably in different environments and it is usually present as aluminosilicate mineral pollucite ($(Cs,Na)_2Al_2Si_4O_{12}\cdot 2H_2O$) (White and Broadley, 2000). Soils and sediments generally contain between 0.3 and 25 mg Cs kg^{-1}, while Cs is more diluted in aquatic ecosystems with concentrations ranging from approximately 0.01 – 1.2 mg L^{-1} in freshwater habitats to approximately 0.5 – 2.0 mg L^{-1} in marine habitats (Komarov and Bennett, 1983, White and Broadley, 2000). Only in areas containing Cs-rich pollucite ores, such as those found in southeastern Manitoba (Teertstra *et al.*, 1992), might Cs cause environmental toxicity (White and Broadley, 2000). However Cs contamination of soil with radionuclides is a worldwide problem. These isotopes arise from the manufacture and testing of thermonuclear weapons and from intentional and unintentional discharges from nuclear installations. The two radioisotopes of Cs (^{134}Cs and ^{137}Cs) are of environmental concern due to their emissions of harmful β and γ radiation, relatively long half-lives, and rapid incorporation into biological systems (White and Broadley, 2000). Contamination of soil with ^{137}Cs deserves special attention because of its high fission yields, long half-life ($t_{1/2}$=30.2 years) and influence on human health (Dushenkov, 2003).

Until today, no essential biological role of stable Cs has been elucidated, although trace quantities have been detected in many organisms (Ghosh *et al.*, 1993). Moreover,

the chemical similarity of Cs(I) and other alkali monovalent cations, in particular K, is the key factor that governs the high mobility of Cs(I) in biological systems (Avery *et al.*, 1991; Davis, 1963; Komarov and Bennett, 1983). Because of the chemical similarity between Cs^+ and K^+, root uptake mechanisms cannot differentiate between these elements easily (White and Broadley, 2000).

Hampton *et al.* (2004) suggest that Cs intoxication in *Arabidopsis thaliana* results from competition between K and Cs for K^+-binding sites on essential proteins. As this is the same toxicity mechanism proposed for Tl, interacts with vital K^+-binding sites in proteins can be the common way of monovalent K similar cation toxicity. Bystrzejewska-Piotrowska and Urbana (2003) have shown that Cs accumulation in leaves of *Lepidium sativum* disturb water uptake, tissue hydration, production of biomass and gas exchange parameters. Stress in PSII was observed as a late stress effect as well. Finally a polymorphism, affecting amino acids close to the K-pore of one Candidate QTL locus, Cyclic nucleotide gated chanel 1 (CNGC1), was identified in *Arabidopsis thaliana* Sorbo (Kanter *et al.*, 2010).

1.5. Population genetics of pseudometallophytes

The evolution of metal tolerance has occurred at different magnitudes in a wide variety of plant species. Studies that have measured both tolerance and hyperaccumulation ability, as judged from screening on a uniform substrate, (Meerts and Van Isacker, 1997; Bert *et al.*, 2000; Escarré *et al.*, 2000; Assunção *et al.*, 2001) generally suggest that while tolerance is positively correlated with soil metal concentration, the ability to hyperaccumulate does not show a significant positive correlation with either. This is one line of evidence suggesting that hyperaccumulation is not controlled by the same mechanism as tolerance (Pollard *et al.*, 2000). However the relationship between tolerance and hyperaccumulation may not be similar for all metals, or for all species.

Boyd and Martens (1992) summarized five hypotheses regarding the possible adaptive benefits of metal hyperaccumulation: (i) a mechanism for increased tolerance, (ii) metal-based allelopathy, (iii) drought resistance, (iv) an “inadvertent uptake” hypothesis: (Boyd and Martens, 1998), or (v) defence against herbivore and pathogen attack. These hypotheses are not necessarily mutually exclusive, although it is not clear how the first three hypotheses could be adaptive on nonmetalliferous soil.

Variation within populations is the level at which natural selection might act to favour

the evolution of hyperaccumulation, if individuals accumulating more metals have greater reproductive success (Pollard *et al.*, 2000). As a matter of fact, different behaviours at the population level have been recorded, providing a paradigmatic example of microevolution (Richau and Schat, 2009). Therefore it appears that although the ability to hyperaccumulate is largely a pervasive property within species, significant genetic variation in hyperaccumulation ability, nevertheless exists, both between populations and within some populations (Pollard *et al.*, 2002). Studies focused on the genotypic differences between and within populations of metallophytes show little evidence of genetic structure and no genetic differentiation between edaphic types, i.e. metallicolous and non-metallicolous populations (Verkleij *et al.*, 1989; Vekemans *et al.*, 1992; Macnair *et al.*, 1999, 2000; Mengoni *et al.*, 2000; Baumbach and Hellwig, 2003; Basic *et al.*, 2006; Baumbach and Hellwig, 2007). This is in agreement with observations that metal tolerances is determined by one or a few single major genes and influenced by a variable number of hypostatic minor genes (Macnair, 1993; Schat *et al.*, 1996; Schat and ten Bookum, 1992; Smith and Macnair, 1998) but the overall genetic variability can still be of comparable levels.

However, it is still likely that the metal-polluted environment and the metalliferous native soils exert selective pressure. Metalliferous habitats are considered as edaphic islands in which this phenomenon is particularly significant and in which genetic adaptation to extreme environmental conditions can be rapid (Antonovics *et al.*, 1971; Ernst, 1974; Bradshaw and McNeilly, 1981; Macnair, 1993). One possible explanation of diverging populations yet variable inside them to the metal tolerance, can be a parapatric speciation model. In the case of *Anthoxanthum odoratum*, several populations, under strong selection for metal tolerance, have diverged from neighbouring non-tolerant populations (on non-metalliferous soil) in tolerance and flowering time and they self-pollinate more frequently. The last two characteristics provide considerable reproductive isolation from adjacent non-tolerant genotypes (Antonovics, 2006).

1.6. *Biscutella laevigata* L.

The perennial herb *Biscutella laevigata* is a European scapose hemicryptophyte with a sporophytic self-incompatibility system (Olowokudejo and Heywood, 1984) belonging to *Brassicaceae* family. Based on the early studies of Manton (1937), *B. laevigata* is thought to have survived the Pleistocene vicissitudes as a diploid (2n=2x=18) in ice-free parts of

the European continent and to have recolonized the Alps as an autotetraploid (2n=4x=36) after the ice ages.

Biscutella laevigata L. subsp. *laevigata* Mant. Pl. Altera (Figure 1.2) is a genetic autotetraploid (with a few diploid populations) with multiple origins involving several diploid progenitors, i.e. the subspecies *B. austriaca* and *B. prealpina* (Tremetsberger *et al.*, 2002). *Biscutella laevigata* subsp. *laevigata* is usually high 18-40 cm, woody at the base, with erect stem usually branched at the top, provided with scattered hairs. Basal leaves are tomentose, grouped in a rosette, spatulate, linear, and entire or ± engraved on the edge (0.7-2 × 3-15 cm). The apex of the leaf is generally acute. Caul leaves are very small, scattered, sessile and partially enclosing the stem. The inflorescence is an

Figure 1.2 – *Biscutella laevigata* L. subsp. *laevigata* Mant. Altera.(a) Alpine morphotype: entire plant (×0.7); petal (×5.4); silique(×5.4); leaves epidermis (×5.4) (originally from Raffaelli and Baldoin, 1997); (b) Flowering; (c) Basal leaves; (d) Siliques.

elongated simple or composed raceme, made up of small yellow flowers with four petals (tetramers), closely obvate, 4-8 mm long, with peduncles of about 1 cm and clear yellowish sepals. The fruit consist of a glabrous, rough, with evident veins silicles formed by two flat, disk-shaped valves (8-14 × 6-8 mm) welded along one side, with a 3-5 mm style.

Chorologically *B. laevigata* is an Alpine and mountain species of southern Europe (Orof.S-Europ.), ranging from Iberian Peninsula to the Balkans and possibly to Caucasus or Anatolia. The anthesis takes place from April to August.

Biscutella laevigata is a pseudometallophyte since it is generally found in non-contaminated areas such as dry meadows, pastures, dry and sunny land and rocks, from 0 to 2,300 m in altitude, and occasionally on metalliferous soils, including Cu, Pb and Zn mine tailings and smelter deposits. *B. laevigata* has been reported to exhibit Cd, Pb and Zn tolerance (Wierzbicka and Pielichowska, 2004) and has also been described as a Cd, Pb and Tl hyperacumulator (Anderson *et al.*, 1999, Wenzel and Jockwer, 1999, Pielichowska and Wierzbicka, 2004). However, to date, nothing is known about physiology of Tl accumulation and the genes involved in the evolution of metal tolerance in *B. laevigata*.¸

1.7. Aim and outline of this thesis

In the first part of this thesis, an ecological survey on metallophytes belonging to the *Thlaspietum cepaeifolii* plant community at the former Pb-Zn Raibl mining site (Julian Alps, Italy) was carried out. The concentrations of Cd, Pb, Tl and Zn were measured in specimens of *Alyssum wulfenianum*, *Biscutella laevigata* subsp. *laevigata*, *Minuartia verna* and *Thlaspi rotundifolium* subsp. *cepaeifolium* collected from native and mining soils, mine tailings and stream banks. *B. laevigata* subsp. *laevigata* was chosen as the most prominent Tl hyperaccumulator although exceptional Tl accumulation has also been found in *A. wulfenianum* and *M. verna* (Chapter 2). Therefore, intra- and inter-specific variations of metal accumulation in other North-Eastern metallicolous and non-metallicolous Italian populations of *B. laevigata* subsp. *laevigata* were tested, as well. Analysis takes into account the metal variability in soil when comparing metallicolous and non-metallicolous populations. The analysis show that a differentiation of a local population of *B. laevigata* subsp. *laevigata* with a Tl tolerance has occurred in the former mining site of Cave del Predil. Moreover a candidate population for Cu tolerance was

found in the metallicolous population from the former mining site of Monte Avanza. Population genetic structure analyses for disentangling selective and demographic history were conducted, as well (Chapter 3). Furthermore, we tested the hypothesis of a population-specific evolution of thallium hyperaccumulation among all *Biscutella laevigata* known populations from distinctly Tl polluted calamine sites, and also checked a non-metallicolous population for its propensity to hyperaccumulate Tl upon supply in a controlled experiment. The metallicolous population Cave del Predil was collected from tailing ponds of a former Pb-Zn Raibl mining site (Tarvisio, Northern Italy); the metallicolous population Les Avinières was collected from tailing ponds of a former Pb-Zn Les Avinières mining site (Saint-Laurent-le-Minier, Ganges, Southern France); the metallicolous population Bolesław was collected from Pb-Zn waste heaps of industrial zone Bukowno (Olkusz, Southern Poland). The non-metallicolous population from Tatra Mountains (Southern Poland) was chosen as a control. Because both, Tl and Cs are K chemical analogues (monovalent cations in solution and of similar ionic radii), we tested the above populations for tolerance and accumulation of Cs, as well. We also compared Tl and Cs accumulation at different K concentrations in the nutrient solution expecting comparable degrees of competitive inhibition by K (Chapter 4).

Because of important features for phytomining it is important to test pseudometallophyte populations for better understanding the physiological, molecular and genetic basis of tolerance and hyperaccumulation. Moreover, phytomining for Cs and Tl could be a very suitable eco-sustainable and economically beneficial activity. Furthermore, Cs and Tl tolerance are not well understood yet. This work gives an important contribution to ecology, genetics and physiology for the understanding of toxic and non-essential elements.

2

Metallophytes and thallium hyperaccumulation at the former Raibl lead/zinc mining site (Julian Alps, Italy)

Guido Fellet, Filip Pošćić, Valentino Casolo, Luca Marchiol

Accepted for publication in Plant Biosystems

2.1. Abstract

An ecological survey on metallophytes belonging to the *Thlaspietum cepaeifolii* plant community was carried out at the former Pb-Zn Raibl mining site (Julian Alps,Italy). The aims of this work were to validate the metal hyperaccumulation and to determine whether hyperaccumulation was restricted to a single element or more. The concentrations of Cd, Pb, Tl and Zn were measured in specimens of *Alyssum wulfenianum*, *Biscutella laevigata* subsp. *laevigata*, *Minuartia verna* and *Thlaspi rotundifolium* subsp. *cepaeifolium* collected from native and mining soils, mine tailings and stream banks. Cadmium hyperaccumulation was not demonstrated, whereas in the case of Pb and Zn the appraisal of hyperaccumulation gave contradictory results. Thallium hyperaccumulation was strongly confirmed in *B. laevigata* subsp. *laevigata* (up to 32,661 mg kg^{-1} of Tl in shoots and bioconcentration factor > 1). Surprisingly this was discovered in *A. wulfenianum* and *M. verna* (up to 1,934 and 3,632 mg kg^{-1} of Tl in shoots respectively, and bioconcentration factor >1). Multiple metal hyperaccumulation was verified in *B. laevigata* subsp. *laevigata* (Pb and Tl), *M. verna* and *T. rotundifolium* subsp. *cepaeifolium* (Pb, Tl and Zn) although it could not be demonstrated in any species when coefficients calculated on the element shoot concentration were considered.

Keywords: Metallophytes, Hyperaccumulators, Thallium, Raibl, Mining site

2.2. Introduction

A few vascular plant species have developed biological mechanisms to tolerate metals and thrive in ecological conditions that are potentially toxic to most plants (Reeves and Baker, 2000).

Metal-tolerant plants acquired specific physiological mechanisms which enabled them to function normally in the presence of high concentration of toxic metals. Depending on the plant species, metal tolerance may result from two basic strategies: metal exclusion – avoidance of metal uptake and restriction of metal transport to the shoots – and accumulation (Baker, 1987).

Broadly defined metallophytes, such plants – *sensu* Lambinon and Auquier (1963) – are divided into: (i) absolute or obligate metallophytes, growing only on metal-contaminated and naturally metal-rich soils and (ii) facultative metallophytes or pseudometallophytes, which grow both on contaminated and non-contaminated soils.

The extreme level of metal tolerance in higher plants is represented by hyperaccumulators. Hyperaccumulators are a restricted group of metallophytes that contain > 100 mg kg^{-1} of Cd, > 1,000 mg kg^{-1} of Ni, Co, Cr, Cu, Pb, > 10,000 mg kg^{-1} of Zn (Baker and Brooks, 1989) and > 500 mg kg^{-1} of Tl (LaCoste *et al.*, 1999) in their tissues. Hyperaccumulation has been reported in a total of approximately 450 plant *taxa* with the highest occurrence among *Brassicaceae* to date (Baker and Brooks, 1989; Reeves and Baker, 2000). On the other hand, in a recent review, Krämer (2010) updated the hyperaccumulation threshold for Tl up to 1,000 mg kg^{-1} and put on discussion the consistence of these threshold values with particular reference to those that designated metal hyperaccumulators based on single plant specimens.

In addition to the metal concentration criterion, the evaluation of the accumulation of potentially toxic trace metals by hyperaccumulators was done using the bioconcentration factor (BF, shoot-to-soil concentration ratio) and the translocation factor (TF, shoot-to-root concentration ratio) (Zhao *et al.*, 2003). In most plants, the BF for heavy metals and metalloids is < 1; in hyperaccumulators, this ratio is always > 1, in some cases reaching 50 – 100 (Ma *et al.*, 2001; Zhao *et al.*, 2003), thus indicating efficiency in metal uptake. Both parameters, rather than arbitrary values of metal concentration, prove that metal hypertolerance is the expression of internal mechanisms of detoxification that evolved as ecological adaptations to edaphic pressures.

More recently, a fourth requirement was proposed (Branquinho *et al.*, 2007): the metal

concentration in the shoots of hyperaccumulators must be at least 10 to 500 times higher than that found in plants from unpolluted soils (e.g. Cd 1 mg kg^{-1}, Pb 5 mg kg^{-1} and Zn 100 mg kg^{-1}, Adriano, 2001).

Althoug the soil metal concentration is the main factor in evolutionary ecology and population dynamics of metallophytes, the genetic variability of plants, expressed by a variety of behavior patterns, plays an important role (Pollard *et al.*, 2002). A number of absolute metallophytes are not hyperaccumulators, whereas others do. Examples of non hyperaccumulators are *Viola calaminaria* and *Armeria maritima* subsp. *halleri* (Brown, 2001; Bizoux *et al.*, 2008). The serpentine endemics *Streptanthus polygaloides*, *Thlaspi montanum* and several species of *Alyssum* are instead, good examples of Ni-hyperaccumulators (Mengoni *et al.*, 2009).

Although typically found on metalliferous soils, *Thlaspi caerulescens* – the most studied hyperaccumulator – is a pseudometallophyte, as populations can also be found on non-metalliferous soils. Similar examples include *T. goesingense*, *T. montanum* and *Arabidopsis halleri* (Reeves and Baker, 1984; Bert *et al.*, 2000).

Mining sites represent a good ecological niche for metallophytes (Batty, 2005). High levels of metals from both geogenic and anthropogenic sources give these sites the potential to support metallophytes and hyperaccumulators. Indeed, hyperaccumulators have been used to identify mineralized rocks and ores (Cannon, 1960).

In Italy, a remarkable example of such environments is the area of the Rio del Lago valley (Julian Alps, NE Italy) where the Pb-Zn mines of Raibl have been worked since ancient times. Ernst (1974) described *Thlaspietum cepaeifolii* Ernst 1965 as a very rare metal plant association strictly related to the mining sites of Raibl. The hyperaccumulator *Thlaspi rotundifolium* subsp. *cepaeifolium* is one of the dominant members of the plant association, which also includes metal resistant ecotypes of *Minuartia verna*, *Silene vulgaris*, *Poa alpina*, *Dianthus sylvestris*, and a series of companion species. The metallophyte *Alyssum wulfenianum* was not included by Ernst (1974) in the composition of the association probably because it was not present in the areas examined. Subsequently, Punz and Mucina (1984) suggested that such endemic plant is also confined to this community.

The soils supporting *T. rotundifolium* subsp. *cepaeifolium* are characterized by Pb content ranging from 600 to 4,000 mg kg^{-1}, Zn content ranging from 7,000 to 140,000 mg kg^{-1} and small amount of Cu, always less than 100 mg kg^{-1} (Ernst, 1974). Reeves and Brooks (1983) reported that similar conditions were recorded in some sites in an area

extending along the Southern Limestone Alps, from the Carnic Alps (Italy) and Gailtal Alps (Austria) eastwards to the Julian Alps (Italy) and Karawanken (Austria/Slovenia), where scattered Pb and Zn metalliferous mineralization and mine workings occur (e.g. Bleiberg ob Villach, and Jaunken, Austria; Črna, Slovenia; Raibl, Italy). This area probably corresponds to the entire distribution of the endemic *A. wulfenianum* and *T. rotundifolium* subsp. *cepaeifolium* (Jalas *et al.*, 1999).

The first evidence of Pb and Zn hyperaccumulation by *T. rotundifolium* subsp. *cepaeifolium* was reported by Reeves and Brooks (1983). They measured a Pb concentration of 8,200 mg kg^{-1} (0.82%) in samples from herbarium specimens collected in the Rio del Lago valley, but did not report any data regarding the soil metal concentrations. As well as Pb, this species was also noted as a Zn hyperaccumulator, with up to 17,300 mg kg^{-1} (1.73%) in its shoots.

This paper reports the results of a field survey conducted in the Rio del Lago valley, with the following objectives: (i) to confirm the presence of the hyperaccumulator *T. rotundifolium* subsp. *cepaeifolium* and *A. wulfenianum*, (ii) to investigate the presence of other metallophytes also defining their metal accumulation potential; (iii) to test the metal hyperaccumulation by the species considered referring to the criteria currently considered in literature; (iv) to verify whether the metal accumulation in these plants is restricted to a single element or more.

2.3. Materials and methods

2.3.1. Soil and plant collecting sites

The village of Cave del Predil (901 m a.s.l., 46° 26′ 26″ N, 13°34′ 16″ E), is situated in the Rio del Lago valley, on the northern side of the Julian Alps (NE Italy) (Figure 2.1).

The minimum and maximum average temperatures at Cave del Predil are 2.4 °C (January) and 16.4 °C (August), respectively. The average annual rainfall is about 2,100 mm (Di Colbertaldo, 1948).

The Raibl mine workings followed the orefields of Pb and Zn, which occurred mainly as sphalerite (PbS) and galena (ZnS). Lower amounts of other sulphides, such as marcasite (FeS_2), pyrite (FeS_2), pirrothine (FeS) and cinnabar (HgS), were also extracted. Moreover, a series of faults within this geological formation has resulted in the occurrence of Pb-Zn sulphide outcrops. The mining activities at Raibl ceased in 1991.

A preliminary investigation was performed in the valley to find out – besides the

endemic hyperaccumulators *Thlaspi rotundifolium* subsp. *cepaeifolium* and *Alyssum wulfenianum* – other species belonging to the *Thlaspietum cepaeifolii* Ernst 1965, the metalliferous plant community ubiquitous at the sampling sites. Such investigation demonstrated that *Biscutella laevigata* subsp. *laevigata* and *Minuartia verna* were ubiquitous at the sampling sites and form a number of distinct subpopulations. Those species are widespread in calcareous talus slopes and pebbly river-beds of the area.

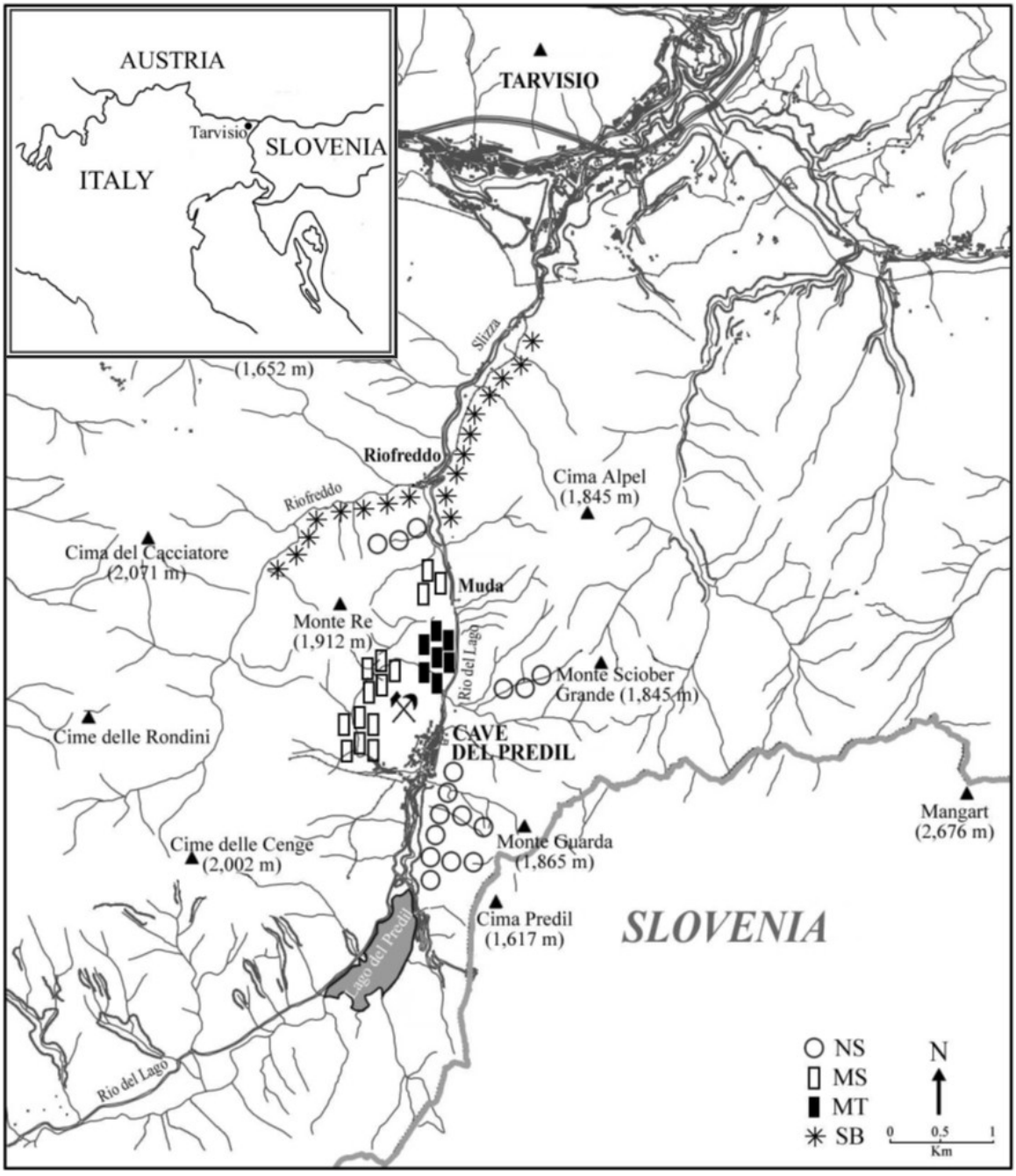

Figure 2.1 – Map showing the location of the collecting sites: native soils (NS, ○), mining soils (MS, □), mine tailings (MT, ▮) and stream banks (SB, ✳).

Indeed, both species had a remarkable capacity to accumulate and tolerate high concentrations of several metals. For these reasons, they were considered in the study.

The pseudometallophyte *B. laevigata* subsp. *laevigata* is a mountain subspecies that also occurs in calcareous dry grasslands on plains. Distributed in Europe from the Eastern Pyrenees to the Transylvanian Alps (Wierzbicka *et al.*, 2004), *B. laevigata* subsp. *laevigata* has previously been recognized, together with *Iberis intermedia*, as a Tl-hyperaccumulator (LaCoste *et al.*, 1999).

Minuartia verna is a South-European mountains distributed species, restricted to dolomitic, serpentine and metalliferous soils in lowland and mountain regions of Central Europe (Verkleij *et al.*, 1989). Antonovics *et al.* (1971) described several subspecies of *M. verna* as strict metallophytes but it has not been reported as a hyperaccumulator, so far.

Plant nomenclature follows the recent checklist of the Italian vascular flora (Conti *et al.*, 2006); Wisskirchen and Haeupler (1998) and Fischer *et al.* (2008) for other species.

Soil and plant samples were randomly collected in the period May – August 2008 at an altitude of 752 – 1,450 m above sea level in the Rio del Lago valley (Figure 2.1). In the surveyed area, four substrate conditions were identified (Figure 2.1): native soil (NS), not impacted by mining operations; mining soils (MS), collected in sites where mine workings were done, mine tailings (MT) and the sediment banks (SB) of the three major alpine streams present in the area (Rio del Lago, Riofreddo, Slizza).

At each plant collecting point a composite bulk soil and rhizospheric soil sample was excavated along the 0 – 20 cm profile. Some soil samples were also collected from other points at which plants were not collected. In total, 135 plant specimens and 142 soil samples were collected and examined.

2.3.2. Soil and plant chemical analysis

Soil samples were dried at room temperature for two days, sieved to < 2 mm and their particle size measured by the Bouyoucos hydrometer method (ASTM, 1972).

Organic carbon (OC%) and nitrogen (N%) content of the substrates were determined following Nelson and Sommers (1996). Samples were first pulverized and up to 15 mg of powder was weighed into silver cups. In order to remove the carbonate content from the sample, HCl (35%) was added to each cup. After drying overnight, each silver cup was placed in a tin cup and wrapped for CHN-analysis (Carlo Erba CHN 1500). The pH of each sample was measured in a soil/water slurry at a 1/2.5 ratio.

The plant specimens were divided into two fractions: root apparatus and aboveground biomass. Plant fractions were rinsed with abundant tap water to remove dust or adhering soil and carefully washed with deionized water.

The soil and plant samples were oven-dried at 105 °C for 24 h and acid-digested in a microwave oven (CEM, MARS Xpress) according to the USEPA 3051 and 3052 method, respectively (USEPA, 1995a,b). After mineralization, both the soil and plant extracts were filtered (0.45 μm PTFE), diluted and analyzed. Total Cd, Cr, Cu, Fe, Ni, Pb, Tl and Zn contents in the extracts were determined by ICP-OES (Varian Inc., Vista MPX). The bioavailable metal fraction of Cd, Pb, Tl and Zn was determined by diethylenetriaminepentaacetic acid (DTPA)/triethanolamine (TEA) according to Martens and Lindsay (1990). The accuracy of the analytical procedure was checked by running the standard solutions every 20 samples. Scandium was used as internal standard.

2.3.3. Data analysis

Before analyzing the data, the Dixon's test was used to verify the presence of outliers in the dataset.

Subsequently, data analysis was performed by univariate statistics using the mean, geometric mean and range values (minimum and maximum), ANOVA and Student-Newman-Keuls test for means comparison. Pearson's correlation coefficient analysis was undertaken to assess the relationship between concentrations of Cd, Pb, Tl and Zn in soils and roots, soil and shoots, roots and shoots. The calculated coefficients were tested for significance with Student-Newmann-Keuls' test according to Costat 6.204 (CoHort Software, Monterey, CA).

Metal concentration in substrates and plants covered a large range of values. Therefore they were presented in figures in a log-log scale. The relationships between the total metal content and DTPA extractable fraction were interpolated with power functions.

2.4. Results

2.4.1. Soil characterization

The general characteristics of the substrates are shown in Table 2.1.

The collected samples were coarse and poor in structure. All soil samples, as well as the mine tailings, were slightly alkaline. The organic carbon (OC) content varied in the range 0.46 – 2.81%. The lowest values were recorded in SB and MT. On the contrary, NS

Table 2.1 – Characterization with physical and chemical parameters and total and bioavailable Cd, Pb, Tl and Zn in native soils (NS), mining soils (MS), mine tailings (MT) and stream banks sediments (SB).

Parameter	NS (n=15)	MS (n=44)	MT (n=36)	SB (n=47)
Sand (%)	73.5(†)±3.1(‡)	72.9±1.2	58.7±0.9	80.8±3.5
Silt (%)	17±1.4	18.3±0.9	28.3±2.3	11.7±0.6
Clay (%)	9.49±2.3	8.69±0.4	13±1.1	7.42±0.4
pH	8.21±0.15	7.84±0.1	8.08±0.1	8.44±0.1
OC (%)	2.81±1.42	0.75±0.15	0.4±0.15	0.46±0.08
Tot N (%)	0.2±0.08	0.07±0.01	0.05±0.02	0.05±0.001
Fe (g kg^{-1})	2.04±0.8	53.2±9.4	44.6±6	14.8±1.7
Cd_{tot} (mg kg^{-1})	0.63(§) (0.37–1.28)(¶)	18.1 (3.64–235)	32.2 (10.4–216)	20.9 (1.74–271)
Pb_{tot} (mg kg^{-1})	33.4 (8.15–176)	4,782 (448–69,386)	7,502 (2,255–59,950)	4,431 (164–36,124)
Tl_{tot} (mg kg^{-1})	161 (120–263)	376 (150–4,549)	424 (164–9,158)	288 (115–862)
Zn_{tot} (mg kg^{-1})	47.6 (10.2–156)	16,930 (2,302–45,513)	18,336 (5,351–66,563)	8,836 (557–33,381)
Cd_{DTPA} (mg kg^{-1})	0.11 (0.02–0.63)	0.73 (0.02–4.37)	1.58 (0.52–15.9)	1.07 (0.16–8.65)
Pb_{DTPA} (mg kg^{-1})	8.14 (1.49–88)	65.9 (8.33–875)	125 (0.01–1,153)	241 (13.2–1,483)
Tl_{DTPA} (mg kg^{-1})	n.d.	1.12 (0.81–7.57)	1.05 (0.01–9.78)	0.21 (0.01–3.82)
Zn_{DTPA} (mg kg^{-1})	9.69 (1.59–71)	411 (83–761)	462 (309–1,486)	260 (49.6–1,050)

(†) Mean; (‡) Mean standard error; (§) Geometric mean; (¶) Range values

that had not been disturbed by any mining activity, had an average OC of 2.81%. Total nitrogen was low (range 0.05 – 0.2%), whereas the highest total Fe concentrations were recorded in the MS and MT samples, as expected (Table 2.1).

The concentration of some trace metals in the soil samples was within the normal range (Adriano, 2001). In particular, the following average concentrations were recorded: 5.94 mg kg^{-1} of Cr, 29.8 mg kg^{-1} of Cu and 6.75 mg kg^{-1} of Ni. Hence, these elements were not considered any further. On the contrary, the concentrations of Cd, Pb, Tl and Zn were anomalous, even though they varied widely.

The total concentrations of Cd, Pb and Zn in NS were not very high. However, a high Tl concentration was found in the soil samples collected outside the mining areas (Table 2.1). Soil samples collected in MS and MT had, as expected, very high metal concentrations. In MS the concentration range values were: Cd 3.64 – 235 mg kg^{-1}, Pb 448 – 69,387 mg kg^{-1}, Tl 150 – 4,549 mg kg^{-1} and Zn 2,302 – 42,513 mg kg^{-1}. MT substrates were characterized by the following range values: Cd 10.4 – 216 mg kg^{-1}, Pb 2,255 – 59,950 mg kg^{-1}, Tl 165 – 9,158 mg kg^{-1} and Zn 5,351 – 66,563 mg kg^{-1}. In spite of the high data variability, it is noticeable that anomalous metal concentrations were also recorded in SB particularly for Pb, Tl and Zn (Table 2.1).

The relationships between total metal content and the amount of the bioavailable fraction in different substrates are shown in Figure 2.2. The data interpolation was done using a power function; this function described the relationship in the case of Cd (R^2=0.68) and Zn (R^2=0.82). This relationship was weaker for Pb (R^2=0.33), whereas there was no relationship between the total content of the element and the bioavailable fraction in the case of Tl (R^2=0.05).

The highest percentages of bioavailability were observed in native soils (on average 28.0%, 23.5% and 21.6% respectively for Cd, Pb and Zn). These percentages dropped below 5% of the total concentration in all the samples collected from the mining areas

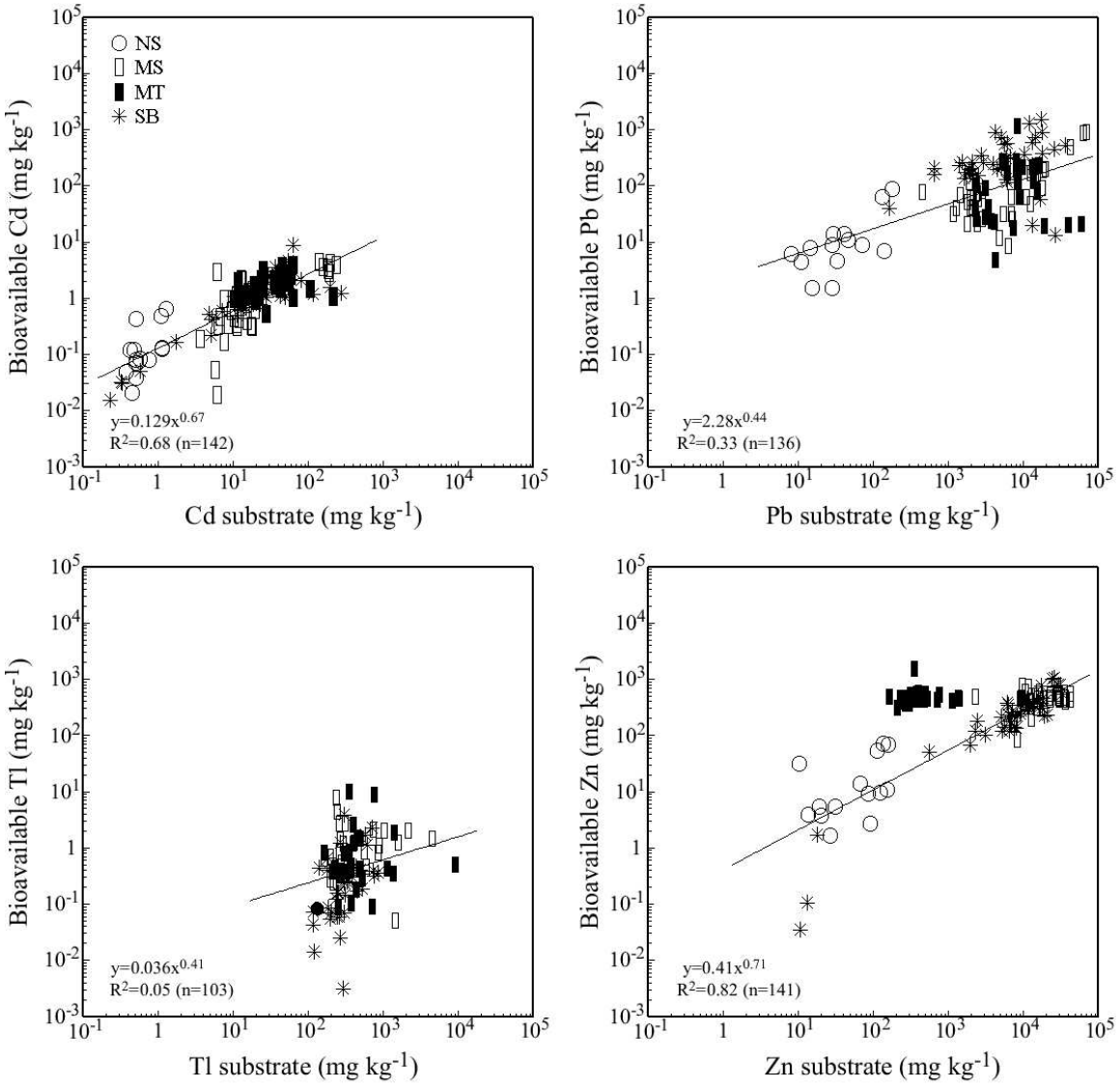

Figure 2.2 – Relationships between total and bioavailable fraction of Cd, Pb, Tl and Zn measured in native soils (NS, ○), mining soils (MS, □), mine tailings (MT, ■), stream banks (SB, ✳).

and stream banks. However, Tl showed a different trend. In several samples, the

bioavailable fraction of this element was not detectable by averaging the data; in fact, the DTPA-extractable fraction was less than 0.1% of the total Tl, confirming the findings of Zbíral *et al.* (2002).

2.4.2. Correlation between soil and plant fraction metal concentrations

Pearson's correlation coefficients between the concentration of Cd, Pb, Tl and Zn in substrates and in plant fractions (roots and shoots) are given in Table 2.2.

Note that given that anomalous concentrations of metals were also found in NS (Tl) and SB (Pb and Zn), the correlation analysis was performed without distinguishing the substrates but considering the entire plant and soil dataset. Overall, there were several cases of significant positive correlation but they were not equally distributed among the species.

In *A. wulfenianum* only the concentration of Tl in roots and shoots correlated positively; neither soil and root, nor soil and shoots correlations were found. The hyperaccumulator *T. rotundifolium* subsp. *cepaeifolium* showed significant root/shoot correlations for all elements, whereas the soil/root relationship was significant only for Cd (Table 2.2). Soil to shoot metal concentrations was correlated for Cd and Pb.

The pseudometallophyte *B. laevigata* subsp. *laevigata* showed a positive correlation for Pb and Zn soil/root concentration. For Cd and Zn, a positive significant correlation was found between the concentration in soil/shoots and roots/shoots (Table 2.2). In *M. verna* the roots/shoots correlation coefficients were positive for all the elements; the same evidence but excluding Tl was verified for soil/roots correlation. Soil/shoot positive correlation was observed for Cd and Pb (Table 2.2).

2.4.3. Plant accumulation of Cd, Pb, Tl and Zn

Figure 2.3 displays the relationship between soil and shoots concentrations on a logarithmic scale.

The closed symbols indicate the hyperaccumulators (*A. wulfenianum* and *T. rotundifolium* subsp. *cepaeifolium*), whereas the open symbols indicate the non-hyperaccumulator species, the strict metallophyte *M. verna* and the pseudometallophyte *B. laevigata* subsp. *laevigata*.

Metal hyperaccumulation were initially evaluated by comparing the metal concentrations in the shoots of the plants with the hyperaccumulation threshold limits. In

Table 2.2 – Pearson's correlation coefficient (r) between metal concentrations in soil, roots and shoots of *Alyssum wulfenianum* (23 specimens), *Biscutella laevigata* subsp. *laevigata* (54 specimens), *Minuartia verna* (33 specimens) and *Thlaspi rotundifolium* subsp. *caepaeifolium* (25 specimens).

Species		Cd	Pb	Tl	Zn
Alyssum wulfenianum	Soil/Roots	0.057^{ns}	0.359^{ns}	0.532^{ns}	0.740^{ns}
	Roots/Shoots	0.574^{ns}	0.139^{ns}	0.001^{**}	0.053^{ns}
	Soil/Shoots	0.406^{ns}	0.487^{ns}	0.527^{ns}	0.848^{ns}
Biscutella laevigata subsp. *laevigata*	Soil/Roots	0.168^{ns}	0.022^{*}	0.467^{ns}	0.000^{***}
	Roots/Shoots	0.018^{*}	0.126^{*}	0.252^{ns}	0.000^{***}
	Soil/Shoots	0.000^{***}	0.584^{ns}	0.350^{ns}	0.040^{*}
Minuartia verna	Soil/Roots	0.000^{***}	0.000^{***}	0.921^{ns}	0.024^{*}
	Roots/Shoots	0.009^{**}	0.006^{**}	0.008^{**}	0.006^{**}
	Soil/Shoots	0.008^{**}	0.032^{*}	0.987^{ns}	0.063^{ns}
Thlaspi rotundifolium subsp. *caepaeifolium*	Soil/Roots	0.000^{***}	0.304^{ns}	0.291^{ns}	0.110^{ns}
	Roots/Shoots	0.000^{***}	0.000^{***}	0.000^{***}	0.000^{***}
	Soil/-Shoots	0.000^{***}	0.034^{*}	0.163^{ns}	0.112^{ns}

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns: not significant

Figure 2.3, these thresholds are indicated by broken lines. Figure 2.3 also shows the 1:1 function, indicating the soil-to-shoot ratio for each element.

The bioconcentration factor ($BF=[Me]_{shoots}/[Me]_{soil}$) and translocation factor ($TF=[Me]_{shoots}/[Me]_{roots}$) were then considered. These coefficients were calculated for the specimens collected at each sampling site. The BF was calculated considering the total metal concentration in soil (Zhao *et al.*, 2003). The average BF and TF are reported in Table 2.3.

Finally, Table 2.4 reports the range values of metal concentration found in root and shoot fractions of our species together with some literature data which will be discussed in the next paper section.

Cadmium. The highest Cd concentrations were found in the shoots of *T. rotundifolium* subsp. *cepaeifolium* (average 14.4 mg kg^{-1}, range 1.35 – 78 mg kg^{-1}, Table 2.3).

The lowest average values were recorded in *B. laevigata* subsp. *laevigata*, which never exceeded the Cd concentration of 2.69 mg kg^{-1}.

None of the species showed a shoot Cd concentration higher than 100 mg kg^{-1} (Figure 2.3). So, the basic condition required to hypothesize hyperaccumulation was lacking. On the other hand, the Cd concentrations in the substrates – certainly anomalous if compared to those in unpolluted soils – were not extreme. Specimens of *M. verna* collected from

MS showed average values of BF and TF > 1 (1.06 and 1.68 respectively). A TF of 1.55 and 1.65 was recorded for *M. verna* (MT) and *T. rotundifolium* subsp. *cepaeifolium* (MS). However, in both cases the corresponding BF were < 1 (Table 2.3).

Lead. The Pb concentrations recorded in plant shoots were in the following ranges: *A. wulfenianum* 28 – 826 mg kg^{-1}, *B. laevigata* subsp. *laevigata* n.d. (not detected) – 874 mg kg^{-1}, *M. verna* 39.9 – 12,574 mg kg^{-1} and *T. rotundifolium* subsp. *cepaeifolium* 29.2 – 2,817 mg kg^{-1} (Table 2.4).

Among the species, *M. verna* showed the highest Pb accumulation in the shoots,

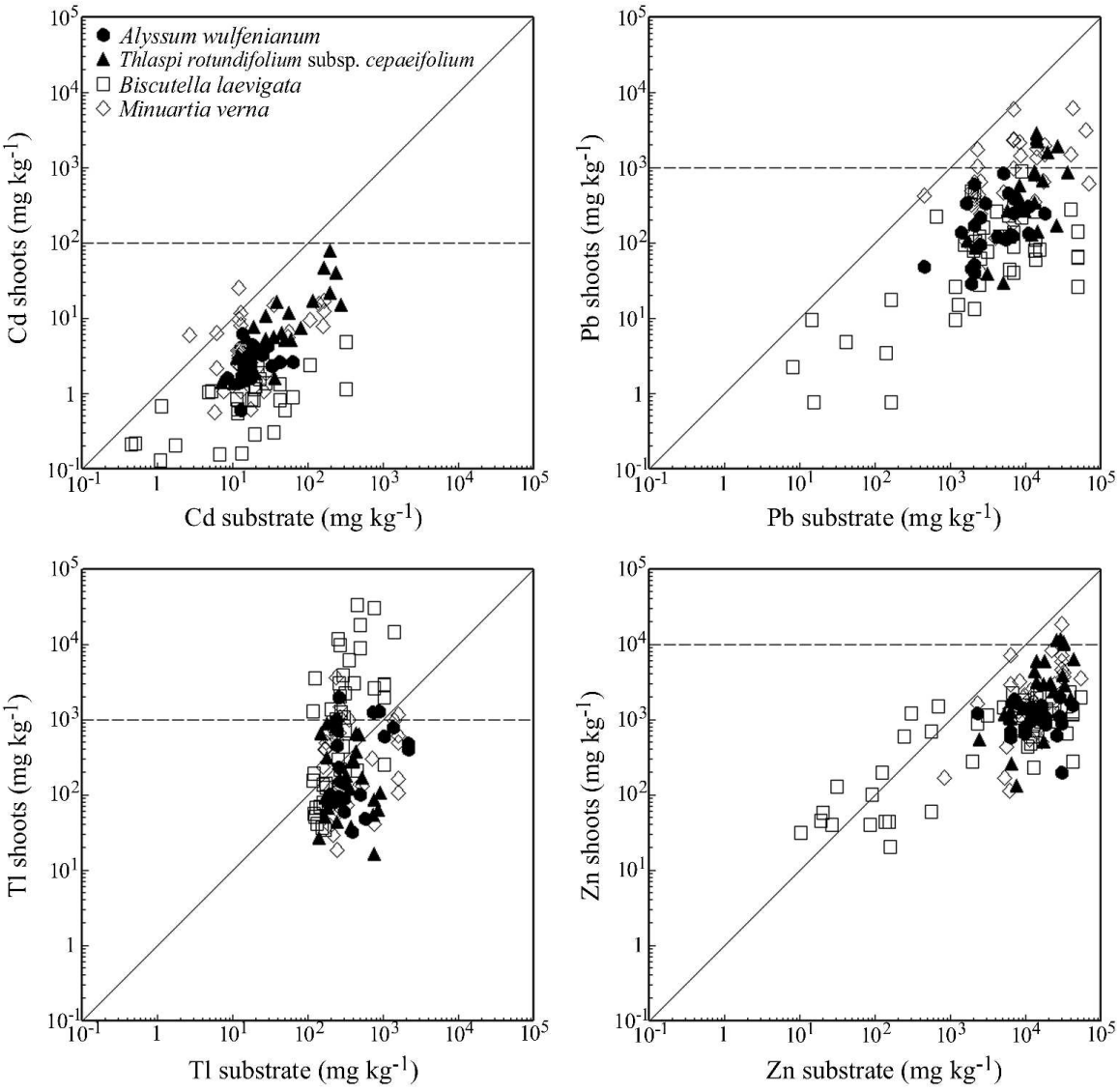

Figure 2.3 – Concentration of Cd, Pb, Tl and Zn in substrates and shoots of *Alyssum wulfenianum*, *Biscutella laevigata* subsp. *laevigata*, *Minuartia verna* and *Thlaspi rotundifolium* subsp. *caepaeifolium* and 1:1 ratio function. Broken lines indicate the hyperaccumulation threshold limits for Cd, Pb, Tl and Zn, respectively (Baker and Brooks, 1989; Kramer, 2010).

Table 2.3 – Bioconcentration factor (BF=[Me]soil/[Me]shoots) and Translocation Factor (TF=[Me]shoots/[Me]roots) calculated for *Alyssum wulfenianum*, *Biscutella laevigata* subsp. *laevigata*, *Minuartia verna* and *Thlaspi rotundifolium* subsp. *caepaeifolium*. Data are mean values.

Species		BF				TF			
		Cd	Pb	Tl	Zn	Cd	Pb	Tl	Zn
Alyssum wulfenianum	MS	0.13a	0.04b	1.98a	0.09a	0.57a	0.23a	0.76a	0.47a
	SB	0.16a	0.11a	0.31b	0.12a	0.88a	0.43a	1.01a	0.95a
Biscutella laevigata subsp. *laevigata*	NS	0.15a	0.1a	0.43b	1.58a	0.8a	0.7a	1.25a	1a
	MS	0.02b	0.02a	5.54b	2.47a	0.36a	8.97a	10.8a	1.88a
	MT	0.04ab	0.13a	27.3a	0.06a	0.44a	2.17a	15.4a	0.74a
	SB	0.07ab	0.08a	4.9b	0.26a	1a	0.73a	7.91a	1.63a
Minuartia verna	MS	1.06a	0.21a	1.95a	0.2b	2.06a	0.77a	1.83a	1.07a
	MT	0.44a	0.33a	3.04a	0.42a	1.55a	0.77a	2.3a	1.34a
	SB	0.14a	0.11a	0.25a	0.11b	0.94a	0.77a	1.02a	1.4a
Thlaspi rotundifolium subsp. *caepaeifolium*	MS	0.28a	0.34a	2.39a	0.16a	1.65a	0.35a	2.24a	0.78b
	MT	0.2a	0.24a	0.58b	0.14a	0.77b	0.24a	0.24a	1.28ab
	SB	0.19a	0.28a	0.4b	0.23a	0.76b	0.28a	2.66a	1.93a

Means with the same letter are not significantly different (p<0.05).

Table 2.4 – Range of concentrations of Cd, Pb, Tl and Zn in plant fractions of *Alyssum wulfenianum*, *Biscutella laevigata* subsp. *laevigata*, *Minuartia verna* subsp. *verna* and *Thlaspi rotundifolium* subsp. *cepaeifolium* found in literature and collected in the mining site of Cave del Predil (Italy).

Species	n(†)	Location	Cd (mg kg^{-1})	Pb (mg kg^{-1})	Tl (mg kg^{-1})	Zn (mg kg^{-1})	Reference
	13	Cave del Predil, Italy (L)(‡)		15–860		31–2,500	Reeves and Brooks, 1983
Alyssum wulfenianum	23	Cave del Predil, Italy (R)	2.20–6.32	253–1,504	n.d.(§)–4,347	354–5,670	(¶)
	23	Cave del Predil, Italy (S)	0.59–5.95	28–826	31.5–1,946	194–1,934	(¶)
	3	Les Aviniéres, France (L)			244–308		Anderson, 1999
	34	Les Aviniéres, France (W)			20–15,199		LaCoste *et al.*, 1999
	15	Tuscany, Italy (W)			<1–2		LaCoste *et al.*, 1999
	2	Gailitz, Austria (S)			295–495		Leblanc *et al.*, 1999
Biscutella laevigata subsp. *laevigata*	1	Arnoldstein, Austria (S)	78.3	1,090		4,870	Wenze and Jockwer, 1999
	5	Mount Prinzera, Italy(#) (W)				53	Lombini *et al.*, 1999
	5	Boleslaw, Poland (S)			0.21		Wierzbicka *et al.*, 2004
	54	Cave del Predil, Italy (R)	n.d.–8.45	n.d.–1,939	n.d.–9,984	34.7–5,692	(¶)
	54	Cave del Predil, Italy (S)	n.d.–4.75	n.d.–874	33.4–32,661	20.2–2,980	(¶)
	n.a.(††)	Bredelem, Germany (L)	49.5	99.5			Ernst *et al.*, 2004
	n.a.	Oker, Germany (L)	42.5	240			Ernst *et al.*, 2004
	n.a.	Pierrefitte, France (W)	<4	62			Reeves *et al.*, 2001
Minuartia verna subsp. *verna*	n.a.	St Dalmas-de-Tende, France (W)	11	14			Reeves *et al.*, 2001
	3	Arnoldstein, Austria (S)	32.6–59	1,290–2,180			Wenze and Jockwer, 1999
	33	Cave del Predil, Italy (R)	0.60–29.2	151–9,329	n.d.–1,286	342–27,521	(¶)
	33	Cave del Predil, Italy (S)	0.55–24.7	40–5,998	18.3–3,632	24.1–18,372	(¶)
	14	Cave del Predil, Italy (L)		130–8,200		2350–7,300	Reeves and Brooks, 1983
Thlaspi rotundifolium subsp. *cepaeifolium*	25	Cave del Predil, Italy (R)	1.30–47.9	282–14,435	35.8–2,275	71.7–11,548	(¶)
	25	Cave del Predil, Italy (S)	1.35–78	29.2–2,817	n.d.–874	28.1–11,573	(¶)

(†) Specimens; (‡) L= leaves, R=roots, S=shoots, W=whole plant; (§) Not detectable; (¶) This work; (#)*Biscutella laevigata* subsp. *prinzerae*; (††) Not available data.

exceeding the hyperaccumulation in 14 specimens out of 33 (42%) (Figure 2.3). The average BF and TF were both < 1 (Table 2.3). About 68% of the specimens (17 out of 33)of *T. rotundifolium* subsp. *cepaeifolium*, which is already known to be a Pb-hyperaccumulator, showed a Pb shoot concentration exceeding the threshold of hyperaccumulation of 1,000 mg kg^{-1} (Figure 2.3). In this case the average BF and TF were both < 1 in all the different substrates without significant differences (Table 2.3).

The average shoot concentration of Pb was quite similar in *A. wulfeniaum* and *B. laevigata* subsp. *laevigata* (221 and 269 mg kg^{-1}, respectively) but the data range was narrower for *A. wulfenianum*. None of the specimens of these species satisfied the conditions of hyperaccumulation regarding shoot metal concentration, BF and TF (Figure 2.3 and Table 2.3).

Thallium. Figure 2.3 shows the variation in the behaviour of hyperaccumulators and non-hyperaccumulators.

Most data from *B. laevigata* subsp. *laevigata* and *M. verna* are distributed above the 1:1 function, being significantly higher than the values recorded for the other species (Figure 2.3). The Tl concentrations observed in the shoots were within the following ranges: *A. wulfenianum* 31.5 – 1,946 mg kg^{-1}, *B. laevigata* subsp. *laevigata* 33.4 – 32,661 mg kg^{-1}, *M. verna* 18.3 – 3,632 mg kg^{-1} and *T. rotundifolium* subsp. *cepaeifolium* n.d. – 14,376 mg kg^{-1} (Figure 2.3).

As expected, being a Tl-hyperaccumulator, a number of specimens of *B. laevigata* subsp. *laevigata* (27 out of 54, 50%) showed a shoot Tl-concentration well above the threshold of 500 mg kg^{-1}. Focusing on the samples collected from MS, MT and SB substrates where the highest Tl concentrations were recorded, this percentage rises to about 63% (27 out to 43) (Figure 2.3). If the higher threshold of 1000 mg kg^{-1} (Krämer, 2010) was considered, then we had respectively about 42% and 53% of specimens exceeding this value. Moreover, the mean BF was 5.54, 27.3 and 4.90 on MS, MT and SB respectively (Table 2.3). In addition, the TF for the same specimens was > 1, ranging from 7.91 (SB) to 15.4 (MT). Note that NS plants showed a TF=1.25 but the BF was < 1 (Table 2.3).

For 8 out of 13 specimens of *A. wulfenianum* collected on MS the Tl concentration in shoots exceeded the threshold of hyperaccumulation (Figure 2.3) and the average BF was 1.98. However, the TF was < 1 (Table 2.3). The values recorded in *M. verna* were very similar – in 13 out of 33 specimens the shoot concentration of Tl was higher than 500 mg kg^{-1}. The average values of BF were 1.95 and 3.04, for plants collected from MS and MT

respectively. The corresponding values of TF were 1.83 (MS) and 2.30 (MT) (Table 2.3).

Finally, some specimens of *T. rotundifolium* subsp. *cepaeifolium* collected from MS also showed a Tl concentration in the shoots that exceeded the hyperaccumulation threshold; the average Tl concentration in shoots was 211 mg kg^{-1} and the highest BF (2.39) was recorded in MS plants. Such value was significantly different than those recorded for MT and SB specimens (0.58 and 0.40, respectively); the correspondent TFs were 2.24 (MS), 0.24 (MT) and 2.66 (SB) (Table 2.3).

Zinc. With regard to the plant shoots, Zn concentration ranges were as follows: *A. wulfenianum* 194 – 1,934 mg kg^{-1}, *B. laevigata* subsp. *laevigata* 20.2 – 2,980 mg kg^{-1}, *M. verna* 24.1 – 18,372 mg kg^{-1} and *T. rotundifolium* subsp. *cepaeifolium* 28.1 – 11,573 mg kg^{-1} (Figure 2.3). The values exceeded 10,000 mg kg^{-1} occasionally in *M. verna* and *T. rotundifolium* subsp. *cepaeifolium* (Figure 2.3).

As for the BF, all the species but *B. laevigata* subsp. *laevigata* scored < 1; the mean BF for *B. laevigata* subsp. *laevigata* specimens from NS and MS were 1.58 and 2.47, respectively. However, the basic requirement for hyperaccumulation was not verified in the shoots. On the other hand, Figure 2.3 clearly shows the capacity for adaptation of this species to a broad range of Zn soil concentrations; the TFs were 1 (NS) and 1.88 (MS), respectively. For all species, with the exception of *A. wulfenianum*, TF was > 1 (Table 2.3), while the other requirements for hyperaccumulation were not fulfilled.

2.5. Discussion

Even though mining activities at Raibl ceased about 20 years ago an issue of severe environmental concern is represented by the lack of any form of management of the mine tailings that cover an area of about 15 ha. These materials are neither stabilized, nor protected and it was verified that pollutants may reach the floodplain through the water flowing downstream.

A survey about the heavy metal content in river sediments of Carinthia (Austria) was conducted by the local Environmental Agency (Müller *et al.*, 1994). The Slizza stream (German name: Gailitz) is a tributary of the river Gail; it crosses the border into Austria and flows through Arnoldstein before entering the Gail. For this reason, the survey considered a sampling point in Italian territory where the highest Zn sediment concentration was found (18,400 mg kg^{-1}). This value, recorded on a sample taken not far from our sampling points, is consistent with our results (mean 8,836 mg kg^{-1}, range 557-

33,381 mg kg^{-1}). The Pb concentration in the same place (7,640 mg kg^{-1}) was the second highest one in the data collected in Carinthia (Müller *et al.*, 1994). Even in this case our data are consistent (mean 4,431 mg kg^{-1}, range 164-36,124 mg kg^{-1}).

The samples of river sediments in Carinthia – and ours – taken from areas without industrial or urban discharges, showed a heavy metal content coherent with both the natural background and the effects of the occurrences in the catchment areas. In our case, the data from the Raibl mining site indicate that there was, or it is still in place, a flow of pollutants from the mine tailings. This was noticed by Reeves and Brooks (1983) who observed that the sediments and the gravels of the Rio del Lago stream have become stained with dark ore-rich material.

Regarding the first objective of this work, the presence of *Alyssum wulfenianum* and *T. rotundifolium* subsp. *cepaeifolium* in the Rio del Lago valley was confirmed. Our data, that include the substrate characterization, allows further evaluation of the metal accumulation in these species. *Alyssum wulfenianum* and *T. rotundifolium* subsp. *cepaeifolium* were often found close to one another in small and isolated ecological niches, on rocky slopes and on sandy stream banks, and particularly only in the most polluted sites where the high levels of metals in the substrates did not allow the establishment of other plants. This explains why the hyperaccumulator data plotted in Figure 2.3 are very similar to that of non-hyperaccumulators. This was particularly evident for Pb and Zn, which were more frequently present in anomalous concentrations in the substrates. On the other hand, when growing in non-metalliferous soils, metal-tolerant individuals are generally less competitive than non-tolerant individuals from adjacent non-metalliferous soils.

Among the species that have so far been listed as hyperaccumulators (Krämer, 2010), the majority are reported to accumulate only one metal (Reeves, 2006). However, the standard for hyperaccumulators has not yet been scientifically defined (Yanqun *et al.*, 2005). Moreover, doubts have arisen recently regarding the most appropriate procedures for sample preparation in order to avoid data misinterpretation. In fact, in the case of plants growing on soil that is highly contaminated with metals, a major concern is the surface contamination of shoot and root tissues by the dust fallout caused by wind erosion (Reeves and Baker, 2000). Furthermore, in sites where mining activities work dust fallout may be originated also by engine traffic and new waste deposits. Another factor able to enhance the risks of plant dust contamination is the plant morphology and in particular the presence of hairs or trichomes on the surface of leaves. It has been reported that in this

case dust can be trapped onto the plant canopies in a form not easy to remove (Faucon *et al.*, 2007).

In our case this problem was carefully considered leading to the following conclusions. First, in the surveyed area the mining activities ceased 20 years ago; Cave del Predil, the company town, has been almost abandoned and therefore currently no human activities cause any kind of disturbance on vegetation. Second, the species that were considered in this study have glabrous leaves and smooth leaf surface; except of *B. laevigata* subsp. *laevigata*, that present, in the metallophytes population, both glabrous and weakly haired individuals. Third, if the plant samples would have been contaminated by dust, an abnormal concentration would have been detected for all metals; this did not happen. Fourth, we tested the dataset for the presence of outliers in particular in those data regarding metal concentration in plant fractions; some records were erased. Taking into account these elements we conclude that our data, although having certain variability, did not suffer from any form of uncontrolled disturbance.

The appraisal of hyperaccumulation was one of the aims of this work. Most of currently known hyperaccumulators have been discovered by surveying herbarium specimens. These basic works have yielded a number of concentration values which are always cited in papers and recent surveys dealing with hyperaccumulation (Reeves and Baker, 2000; Reeves, 2006).

The second aim of this work was to find out in the Rio del Lago valley other metallophytes than *A. wulfenianum* and *T. rotundifolium* subsp. *cepaeifolium* and to define their metal accumulation potential. *Minuartia verna* and *B. laevigata* subsp. *laevigata*, unlike the hyperaccumulators studied by Reeves and Brooks (1983), were collected from substrates with normal or slightly high metal concentrations and from anomalous ones, which confirms the typical behavior of pseudometallophytes. The metal concentrations in the soil and plant fractions were correlated, demonstrating that the metal tolerance in these species is not based on metal exclusion but on specific adaptation to the ecological conditions of metalliferous soils. The first species is a very common species on metalliferous soils. It has been recorded at a number of mine sites in Europe where studies on metallophytes were performed (Smith, 1979; Ernst *et al.*, 2004; Wenzel and Jockwer, 1999). The second species, *B. laevigata* subsp. *laevigata*, is known for Tl-hyperaccumulation; we therefore carried out a literature survey to find data on metal accumulation/hyperaccumulation by *B. laevigata* subsp. *laevigata*, in order to compare our results. Table 4 reports literature data for all the species considered.

Since hyperaccumulation is defined as uptake and sequestration of exceptional concentration of an element in the aboveground parts of a plant under field conditions (Pollard, 2000), in the evaluation of hyperaccumulation, the relationship between metal concentration in soil and in the plant shoots is very important. This relationship is missing when the data on the soil is not available, as in the case of herbarium specimens.

As previously mentioned, the appraisal of hyperaccumulation in our species took place considering four requirements: (i) metal concentration threshold limits, (ii) BF, (iii) TF and (iv) reference to metal concentration in plants from normal soils.

When only the metal concentrations in the shoots of plants were compared to the threshold limits for the metals, evidences of Tl-hyperaccumulation resulted for all the four species. Several specimens of *A. wulfenianum*, collected from the soils polluted by the mining operations, revealed Tl shoot concentrations well above the hyperaccumulation proposed by LaCoste *et al.* (1999); whereas only in 5 specimens the threshold by Krämer (2010) was exceeded. No literature references have been found to support this observation that, to our best knowledge is the first one. A high intraspecific variability disturbed the data regarding *T. rotundifolium* subsp. *cepaeifolium*. However, some cases of high Tl shoot concentration were recorded and only partially supported by the BF.

In *M. verna* the conditions for Tl-hyperaccumulation were fulfilled; also in this case, no references of Tl accumulation were found.

Thallium is listed by USEPA as one of the 13 priority pollutants (Keith and Telliard, 1979). In soils not exposed to anthropogenic pollution, Kabata-Pendias (2001) reported a Tl concentration within the range 0.02-2.8 mg kg^{-1}. Most of crops grown in non-contaminated soils had Tl contents comprised in the range 0.02-0.04 mg kg^{-1}. Values of 2 to 7 mg kg^{-1} are considered anomalous (Adriano, 2001).

The results of an extensive investigation on Tl-hyperaccumulation were reported by LaCoste *et al.* (1999). So far, the highest Tl concentration recorded in plants (15,199 mg kg^{-1}) was observed in *B. laevigata* subsp. *laevigata*. In our dataset this value was more than doubled (32,661 mg kg^{-1}).

Lead hyperaccumulation, based on metal shoot concentration, was verified for *M. verna* and *T. rotundifolium* subsp. *cepaeifolium*. In this last species, that was the first Pb-hyperaccumulator discovered, the shoot of several specimens exceeded the Pb threshold limit of 1000 mg kg^{-1}. The BFs for both the species did not support the supposed Pb-hyperaccumulation. This evidence seems to weaken the historical reference by Reeves

and Brooks (1983) but at the same time it suggests the necessity of combining the element concentration in the plant shoots with ecological data (i.e. metal concentration in the rhizospheric soil).

Only *M. verna* and *T. rotundifolium* subsp. *cepaeifolium* showed shoot metal concentration-based hyperaccumulation of Zn. Also in this case the hyperaccumulation for both metals was not confirmed adding the BFs.

In our work, actually, the fourth requirement was not fully verified for all species because we lacked data references from unpolluted sites. The endemic *A. wulfenianum* and *T. rotundifolium* subsp. *cepaeifolium* and the strict metallophyte *M. verna* colonize metalliferous soils and are not present in normal or unpolluted soil. To some extent, we considered this requirement as being verified only for *B. laevigata* subsp. *laevigata*, a pseudometallophyte whose populations are also widespread at unpolluted sites.

The last aim of this paper was to determine whether metal hyperaccumulation in the species studied is restricted to a single element or more. It was demonstrated that this depends on the method with which hyperaccumulation is evaluated. Without considering BF and TF, multiple hyperaccumulation was verified in *B. laevigata* subsp. *laevigata* (Pb and Tl), *M. verna* and *T. rotundifolium* subsp. *cepaeifolium* (Pb, Tl and Zn). On the contrary, when coefficients which consider the concentration in shoots were considered, then multiple hyperaccumulation could not be demonstrated in any species.

2.6. Conclusions

Overall, the species observed in our survey showed a high metal resistance being able to colonize and survive in substrates strongly contaminated by the mining activities.

This strong metal tolerance must be based on physiological mechanisms of detoxification since they are not metal excluders. It is likely that the plant populations that have developed in this valley are highly adapted to the local environmental conditions: climate, altitude and soil fertility, but most of all to metal outcrops and the metal contamination in the soils and sediments.

The most relevant result provided in this paper is that even with the most restrictive evaluation currently accepted, Tl-hyperaccumulation was strongly confirmed in *Biscutella laevigata* subsp. *laevigata* and demonstrated for *Minuartia verna* and *Thlaspi rotundifolium* subsp. *cepaeifolium*.

Metal tolerance in pseudometallophytes is an elegant example of microevolution

driven by anthropogenic activities and is among the most well-studied phenomena in evolutionary biology (Bradshaw and McNeilly, 1981; McNair, 1987). This leads to the conclusion that the ecological pressure by metalliferous habitats is highly meaningful in terms of biodiversity. Moreover, this suggests that biodiversity conservation strategies should focus not only on native environments but also on environments altered by anthropogenic impacts (Whiting *et al.*, 2004; Becker and Dierschke, 2008; Stevanović *et al.*, 2010).

Since a plant's metal tolerance is the result of a genotype × environment interaction (Pollard *et al.*, 2002), it is appropriate to integrate ecological and genetic data when studying metallophytes (Ouborg and Vriezen, 2007; Roosens *et al.*, 2008).

Further work is currently in progress to pinpoint the genetics and morphological traits of the populations of metallophytes at the Raibl mining site, with particular reference to *Biscutella laevigata* subsp. *laevigata*, combining ecological data with genomics. Merging these approaches should provide new information and possibilities for the selection of plants suitable for phytotechnologies.

Acknowledgements

This research was part of the project ''Persistent green capping on polluted or metalliferous sites: functional role and reservoir of biodiversity''supported by the Italian Ministry of University and Research (PRIN 2008, N° 2008JZ27T7). The authors are indebted to Rufus Chaney (USDA-ARS, Beltsville, MD, USA) for his valuable comments on the manuscript and to Antonino Danelutto for giving the locations of populations of *Alyssum wulfenianum* and *Thlaspi rotundifolium* subsp. *cepaeifolium* in the Rio del Lago valley. Technical assistance by Diego Chiabà and the contributions made by Sabina Moroldo are gratefully acknowledged.

3

Ecological and genetic differentiation at regional scale among metallicolous and non-metallicolous populations of the pseudometallophyte *Biscutella laevigata* subsp. *laevigata*

Filip Pošćić, Guido Fellet, Valentino Casolo, Luca Marchiol and Massimo Vischi

Submitted to Plant Ecology and Diversity

3.1. Abstract

When pseudometallophytes colonize metalliferous soils a selection favoring the metal-tolerant genotypes occurs.

We investigated intraspecific variation of metal accumulation in populations of *Biscutella laevigata* subsp. *laevigata*. Tl tolerance, hyperaccumulation traits and genetic differentiation of populations were analyzed.

Plant specimens were collected in NE Italy. The rhizospheric soils and plant fractions were analyzed for metal concentrations. Thallium tolerance was examined in populations from uncontaminated soil and mine tailings in which local adaptation was expected. The genetic structure of populations was investigated using Amplified Fragment Length Polymorphism (AFLP) markers with a band-based approach.

The maximum Cu concentration – 252 mg kg^{-1} – was recorded in the shoots of *Ava* specimens, whereas the highest concentration ever found in plants, up to 32,661 mg kg^{-1} of Tl in shoots was recorded in *Cap* population. Moreover, *Cap* population was clearly differentiated in cluster analysis.

The data support the novel hypothesis that a differentiation of a local population of *B. laevigata* subsp. *laevigata* with a Tl tolerance has occurred in the former mining site of Cave del Predil.

Keywords: *Biscutella laevigata* subsp. *laevigata*; heavy metals; thallium tolerance; hyperaccumulation; Amplified Fragment Length Polymorphism (AFLP)

3.2. Introduction

Metallophytes are a small group of higher plant species that are adapted to metalliferous or heavy metal-contaminated soils. A more restricted group of metallophytes named hyperaccumulators have the ability to accumulate high metal concentrations in their aerial parts (Baker and Brooks, 1989).

Biscutella laevigata L. (Brassicaceae) is a European hemicryptophyte with a sporophytic self-incompatibility system (Olowokudejo and Heywood, 1984). Based on the early studies of Manton (1937), *B. laevigata* is thought to have survived the Pleistocene vicissitudes as a diploid (2n=2x=18) in ice-free parts of the European continent and to have recolonized the Alps as an autotetraploid (2n=4x=36) after the ice ages. The subspecies *laevigata* is a genetic autotetraploid with multiple origins involving several diploid progenitors, i.e. *B. laevigata* subsp. *austriaca* and *B. laevigata* subsp. *prealpina* (Tremetsberger *et al.*, 2002). With regards to plant ecology, *B. laevigata* is a pseudometallophyte with populations on both metalliferous and non-metalliferous soils, and has also been described as a Tl hyperaccumulator by Anderson *et al.* (1999). However, previous works of Anderson *et al.* (1999), LaCoste *et al.* (1999), Leblanc *et al.* (1999) and Escarré *et al.* (2010) on supposed Tl tolerance and hyperaccumulation are based mostly on mere analysis of elemental concentrations in plants harvested at the various contaminated field sites, rather than on an analysis of metal tolerance per se. For highlighting evidence of plant local adaptation to their particular soils it is therefore necessary to provide analyzes that take into account the metal variability in soil when comparing metallicolous and non-metallicolous populations. Finally, proving adaptation (differential tolerance and/or differential accumulation behavior) of metallicolous genotypes would require tolerance test in controlled conditions (Schat and ten Bookum, 1992).

The present study investigated (i) metal (hyper)accumulation and (ii) thallium tolerance, of different populations of *B. laevigata* subsp. *laevigata* from North-East Italy. Moreover, a preliminary (iii) analysis of population genetic variation was carried out by Amplified Fragment Length Polymorphism (AFLP) markers.

3.3. Materials and Methods

3.3.1. Collection of plants and soils

Specimens of *Biscutella laevigata* subsp. *laevigata* belonging to 15 populations were

collected from June to August 2008 and 2009 in Friuli Venezia Giulia (I) and Veneto (I) (Figure 3.1). The sampling sites were selected according to the *specimina visa* reported by Raffaelli and Baldoin (1997) and information on locations of *B. laevigata* subsp. *laevigata* specimens from the Museo Friulano di Storia Naturale of Udine. Given that the aims of this paper were to investigate the possible differences in the metal accumulation ability of populations of *B. laevigata* subsp. *laevigata* and their genetic structure, some populations were collected from former mining sites (Table 3.1), where it was assumed that anomalous concentrations of metals would be found in the soil. Table 3.1 gives a brief habitat description of each of the sampling sites. At all sites, plants were collected together with a sample of rhizospheric soil from a maximum depth of 20 cm. The populations were each represented by 12–27 specimens.

The *Ava* population was collected from the vicinity of the former copper mine of Monte Avanza, Forni Avoltri (Carnic Alps). The *Cap* population was sampled at the former lead-zinc mining area of Cave del Predil, Tarvisio (Julian Alps). Specimens of the *Sal* population were collected at the former lead-zinc mine of Salafossa, San Pietro di Cadore (Carnic Alps).

3.3.2. Soil and plant analyses

Soil samples were dried at room temperature for two days and sieved to<2 mm. The pH of each sample was measured in soil/water slurry at a 1/2.5 ratio. The soil samples were oven-dried at 40 °C for 24 h and then ground to a powder; up to 10 mg of powder

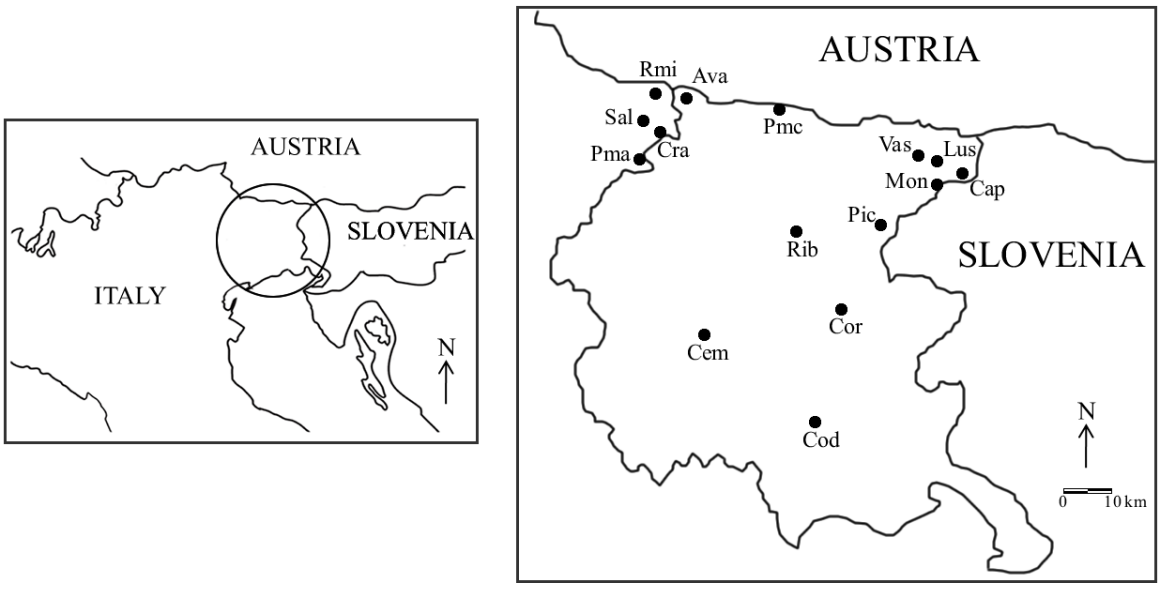

Figure 3.1 − Locations of the sample collection sites.

Table 3.1 – Location, habitat description, CORINE biotope codes and definitions (Commission of the European Communities 1991) of plant and soil sampling sites.

Site	Samples	Habitat description	Altitude	Latitude	Longitude
	n		m	N	E
Monte Avanza – *Ava*	18	Anthropic site, former Cu mine site – 87.2 Ruderal communities	1,750	46° 37′ 17″	12° 45′ 15″
Cave del Predil – *Cap*	16	Anthropic site, former Pb-Zn mine site – 87.2 Ruderal communities	901	46° 26′ 26″	13° 34′ 16″
Cellina-Meduna – *Cem*	14	Natural site, pebble river-bed site – 34.75 Eastern sub-Mediterranean dry grasslands	59	46° 00′ 49″	12° 47′ 30″
Codroipo – *Cod*	12	Semi-natural site, meadow – 34.75 Eastern sub-Mediterranean dry grasslands	47	45° 58′ 30″	12° 56′ 15″
Cortale – *Cor*	22	Semi-natural site; meadow – 34.75 Eastern sub-Mediterranean dry grasslands on calcareous soil	161	46° 09′ 21″	13° 15′ 20″
Casera Razzo – *Cra*	15	Semi-natural site; mountain pastures – 36.5 Alpine and sub-alpine fertilized grassland	1,765	46° 28′ 46″	12° 35′ 37″
Monte Lussari – *Lus*	20	Limestone slopes of Monte Lussari, anthropic site, forest track side – 87.2 Ruderal communities	1,736	46° 28′ 60″	13° 31′ 31″
Altopiano Montasio – *Mon*	25	Semi-natural site; mountain pastures – 36.5 Alpine and sub-alpine fertilized grassland	1,503	46° 24′ 34″	13° 26′ 15″
Pian dei Ciclamini – *Pic*	23	Natural site, gravel bars in the Mea stream – 24.22 Vegetated river gravel banks	755	46° 18′ 17″	13° 18′ 57″
Passo della Mauria – *Pma*	20	Anthropic site, gravel car park – 87.2 Ruderal communities	1,305	46° 27′ 13″	12° 30′ 50″
Passo Monte Croce Carnico – *Pmc*	25	Semi-natural site; mountain pastures – 36.5 Alpine and sub-alpine fertilized grassland	1,369	46° 35′ 59″	12° 56′ 20″
Rivoli Bianchi – *Rib*	27	Natural site, limestone slope – 34.75 Eastern sub-Mediterranean dry grasslands	270	46° 19′ 09″	13° 08′ 18″
Rio della Miniera – *Rmi*	18	Natural site, banks of Miniera stream – 24.22 Vegetated river gravel banks	1,452	46° 35′ 34″	12° 43′ 09″
Salafossa – *Sal*	20	Anthropic site, former Pb-Zn mine site – 87.2 Ruderal communities	1,108	46° 34′ 43″	12° 37′ 18″
Val Saisera – *Vas*	17	Natural site, banks of Saisera stream – 24.22 Vegetated river gravel banks	852	46° 28′ 14″	13° 29′ 39″

were weighed and hydrochloric acid (18%) was added to each sample to remove the carbonate content. The organic carbon (OC) and nitrogen (N) contents were determined by an Elemental Analyzer (Carlo Erba CHN 1500).

The plant specimens were divided into roots and shoots. Plant fractions were rinsed with tap water to remove adhering soil and dust and carefully washed with deionized water. The soil samples and plant fractions were then oven-dried at 105 °C for 24 h and acid-digested according to EPA 3051 and 3052 methods respectively (USEPA, 1995a, b). Total contents of Cd, Cr, Cu, Ni, Pb, Tl and Zn in soil and plants extracts were determined by an ICP-OES (Varian Inc., Vista MPX). The total Fe concentration in soil samples was also determined. The accuracy of the analytical procedure was checked by running standard solutions every 20 samples. Scandium was used as the internal standard. For the statistical analysis, in those samples where an element was not detected, its concentration was assumed to be one-half of the respective limits of detection (Nadal *et al.*, 2004). Detection limits were: 0.2 μg L^{-1}, 0.5 μg L^{-1}, 0.9 μg L^{-1}, 0.3 μg L^{-1}, 0.7 μg L^{-1}, 1.5 μg L^{-1}, 2.0 μg L^{-1} and 0.2 μg L^{-1} for Cd, Cr, Cu, Fe, Ni, Pb, Tl and Zn respectively.

3.3.3. Thallium tolerance test

Cap population and *Cod* population (as the representative of all the other non-metallicolous populations) were chosen for the tolerance test. Twenty days from germination the seedlings with fully expanded primary leaves were transferred to an aerated modified Hoagland's pre-culture solution composed of 2 mM KNO_3, 1 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4, 0.50 mM $MgSO_4$, 10 μM $FeSO_4$, 3.50 μM $MnCl_2$, 11.50 μM H_3BO_3, 0.30 μM $ZnSO_4$, 0.10 μM $CuSO_4$ and 0.03 μM Na_2MoO_4 in demineralized water and pH 6.0 adjusted. Plants were grown in a growth chamber (12-h PAR 500 μmols $m^{-2}s^{-1}$, 25/22 °C day/night, relative humidity 75%).

After acclimating for a week, the seedlings were exposed to a series of Tl (0, 0.1, 1, 10, 100 mg L^{-1} $TlNO_3$) concentrations (5 plants per population per concentration as replicates), in a background solution of the same composition as the pre-culture solution. Before the treatment period the root tips were stained black in a stirred suspension of finely powdered active carbon to facilitate the measurement of root growth (Schat and ten Bookum, 1992). After 5 days of exposure, the length between the black zone and the root tip, representing the root elongation, was measured (Schat and ten Bookum, 1992). Tl concentrations were determined in roots and shoots. Root material was carefully washed with deionized water and then blotted with tissue paper. The plant fractions were then

oven-dried at 105 °C for 24 h and acid-digested according to EPA 3052 method (USEPA, 1995b). Total content of Tl in plants extracts were determined by an ICP-OES (Varian Inc., Vista MPX). The accuracy of the analytical procedure was checked by running standard solutions every 20 samples. Scandium was used as the internal standard.

3.3.4. Amplified Fragment Length Polymorphism analysis

The genomic DNA of the samples was extracted from young leaves and then stored at -80 °C, according to Doyle and Doyle (1990).

Amplified Fragment Length Polymorphism (AFLP) analysis was conducted using the protocol described by Zabeau (1993) and Vos *et al.* (1995) with slight modifications: 600 ng of genomic DNA were digested with 5U of PstI and 5U of MseI enzymes for 3.5 h; the selective amplification was carried out using two primer combinations Pst55/Mse47 and Pst55/Mse62. These primer combinations were chosen as the most polymorphic after a preliminary experiment with several primer combinations. After selective amplification, the AFLP fingerprints were scored with an Applied Biosystem 3730 DNA sequencer using ROX 500 as the size standard.

3.3.5. Data analysis

The soil and plant data were analyzed using CoStat version 6.20 (CoHort Software). Dixon's test was used to test the presence of outliers in the dataset. A test of normal distribution and homogeneity of variance was performed in order to choose the proper statistical tools. The results of the test suggested the use of non-parametric statistics. Non-parametric bivariate correlations coefficients (Spearman's test) were used when calculating correlations between metal concentrations in the soils and in the plant roots and shoots. Statistical differences among soil groups, with particular reference to the concentrations of Cu, Pb, Tl and Zn, were analyzed using the Kruskal–Wallis test.

The relationships between concentrations, either in soil and shoots or roots and shoots per plant, were provided with regression curves and coefficients on power functions for edaphic types (metallicolous and non-metallicolous populations).

To adjust the metal concentrations in shoots for metal concentrations in soil or roots, non-parametric one-way ANCOVA (Conover and Iman, 1982) was used, with shoots metal concentrations as the dependent variable, either soil or roots metal concentrations as the covariate, and edaphic types as the main factor. In order to detect if some metallicolous populations present a significantly higher slope when compared to the other

metallicolous populations we also used a non-parametric ANCOVA within metallicolous populations. Fisher LSD post hoc Bonferroni corrected was performed for multiple comparisons within metallicolous populations.

The Index of Tolerance (IT), based on the root elongation data of Tl tolerance test, was calculated following Schat and ten Bookum (1992). A test of normal distribution and homogeneity of variance was performed in order to choose the proper statistical tools. The results of the test suggested the use of parametric statistics. Statistical differences among the two populations for IT and Tl uptake in roots and shoots were analyzed using two-way analysis of variance followed by a Fisher LSD post hoc Bonferroni corrected for multiple comparisons.

The DNA fingerprints were analyzed with Gene Mapper version 3.0 (Applied Biosystem), based on the presence or absence of peaks between 50–500 bp in size. The resulting AFLP peak patterns were manually scored as dominant markers for the presence (1) or absence (0) of peaks. Bands of equal fragment size were interpreted as homologous. Only distinct polymorphic peaks were scored. To filter out the background, peaks of less than 100 fluorescence units were not scored. Markers with evidence of false peaks (small, unscoreable peaks in a size bin in which other samples had larger scoreable peaks) were discarded from all samples. To prevent scoring of peaks missing due to DNA degradation rather than genetic variation we determined the final marker size range according to the last detectable monomorphic marker even if it was smaller than the 500 bp upper size limit. To calculate the error rate (Bonin *et al.*, 2004) of the AFLP data, 8% of the samples were extracted twice. The AFLP profiles of the replicates were scored and compared with each other.

AFLP data were analysed with a band-based approach (Bonin *et al.*, 2007) because of the lack of Hardy-Weinberg equilibrium due to the polyploid genetic structure of *B. laevigata* subsp. *laevigata* (Parisod and Christin, 2008). Data were compiled in a binary matrix and relative genetic distance was estimated according to Nei and Li (1979). The binary matrix was inferred by a bootstrap analysis of 1000 replicates. The genetic distance matrices were compiled with the phylogenetic inference package PHYLIP version 3.6 (Felsenstein 2005) using the Neighbor and Consense options for constructing a neighbor-joining tree (Saitou and Nei, 1987). The tree file was visualized and manipulated using the software MEGA version 5 (Tamura *et al.*, 2011).

Genetic differentiation among populations and among individuals within populations, between and within edaphic types (metallicolous vs. non-metallicolous), within each

edaphic group among populations and among individuals within populations, were estimated by the Analysis of Molecular Variance (AMOVA, Excoffier *et al.*, 1992) procedure based on the squared Euclidian distance. The variation between populations was partitioned into pairwise Φ_{ST} distances between populations to examine their relative contribution to the total molecular diversity. Analysis of Molecular Variance and the pairwise Φ_{ST} distances between populations was performed through Arlequin version 3.5.1.2 (Excoffier and Lischer, 2010). Statistical significance was assessed by 1,023 permutations.

The correlations between the populations pairwise Φ_{ST} distance matrix and the log geographic distance of each population were determined using the Mantel test (Mantel, 1967) option of Arlequin version 3.5.1.2 (Excoffier and Lischer, 2010) with 1,000 random permutations.

3.4. Results

3.4.1. Soil and plant analyses

The pH, organic carbon (OC), total nitrogen (N) and total Fe percentages observed in the collected soils are shown in Table 3.2. For the majority of sites (10 out of 15), the soil pH values were slightly to moderately alkaline (pH range 7.44–8.27) (Soil Survey Division Staff, 1993). Only the *Pic* and *Vas* sites had very strongly alkaline soils, at pH 9.12 and 9.14 respectively. These samples were collected on the gravel banks of two streams and so should be not referred to as soil (Table 3.2). For the same reason, the lowest percentages of OC were recorded in these samples, whereas higher OC percentages were recorded in the samples collected from mountain (*Pmc*) and plain grasslands (*Cod*) (Table 3.2). The soil N content ranged from 0.05 to 0.95%. As expected, the values of OC% and N% observed in the mining site samples were among the lowest (Table 3.2). Iron is one of the major constituents of the lithosphere and its presence in soils is therefore measured as a percentage of the bulk soil. In our study the highest average Fe value was observed in the *Cap* site (4.5%) from which more iron sulphides had been extracted than other minerals. At the other mining sites, the Fe concentrations were at the same level as on the natural sites (Table 3.2); the average Fe concentration ranged between 0.04 and 3.5%.

The thresholds given by Italian legislation for residential lands are 2, 150 and 120 mg kg^{-1} for Cd, Cr and Ni respectively (Legislative Decree 152/2006). Since the

Table 3.2 – Soil characterization of sampling sites.

Site	pH	OC (%)	Tot N (%)	Fe (%)
Ava	7.51 (0.18)(†)	1.19 (0.51)	0.10 (0.03)	0.55 (0.05)
Cap	8.08 (0.40)	0.46 (0.05)	0.05 (0.01)	4.50 (0.08)
Cem	8.47 (0.34)	2.38 (0.90)	0.22 (0.06)	0.18 (0.00)
Cod	7.79 (0.01)	8.32 (0.82)	0.81 (0.10)	1.98 (0.10)
Cor	8.09 (0.02)	2.43 (0.14)	0.25 (0.01)	1.28 (0.02)
Cra	8.12 (0.01)	4.24 (0.24)	0.29 (0.00)	2.38 (0.15)
Lus	8.38 (0.01)	1.19 (0.08)	0.20 (0.00)	0.20 (0.02)
Mon	7.44 (0.42)	7.38 (0.43)	0.56 (0.04)	2.67 (0.21)
Pic	9.12 (0.05)	-(‡)	0.14 (0.03)	0.04 (0.00)
Pma	8.27 (0.03)	2.42 (0.13)	0.19 (0.01)	0.49 (0.04)
Pmc	7.97 (0.03)	10.1 (0.15)	0.95 (0.01)	3.40 (0.01)
Rib	7.61 (0.02)	3.29 (0.05)	0.37 (0.02)	0.20 (0.00)
Rmi	8.23 (0.21)	3.24 (0.05)	0.20 (0.00)	0.98 (0.14)
Sal	8.53 (0.15)	0.72 (0.10)	0.15 (0.01)	1.67 (0.16)
Vas	9.14 (0.30)	0.37 (0.03)	0.13 (0.03)	0.15 (0.01)

(†) Standard error; (‡) not detectable

concentrations of Cd, Cr and Ni in all soil samples were lower than the upper threshold limits they are not reported in Table 3.3. The total Pb, Tl and Zn concentrations found in mining site samples (*Ava*, *Cap* and *Sal*) were significantly higher than those at other sites; in the case of Cu this was verified only for *Ava* (Table 3.3). The highest values were recorded in the *Cap* samples, with the exception of Cu. In fact, a very high concentration of this element was found in the *Ava* samples with on average 6.1 times higher than the threshold of 600 mg kg^{-1} given by Italian legislation for industrial sites (Legislative Decree 152/2006). This is explained by the fact that Cu sulphates were mainly extracted from the *Ava* mines. The Cu concentration found at other sites ranged from 0.2–42.6 mg kg^{-1}. As a consequence of the activities that took place at *Ava*, *Cap* and *Sal*, we recorded very high concentrations of Pb and Zn in the soil samples. In the case of Pb, on average, they were about 3.9 (*Ava*), 7.7 (*Cap*) and 2.1 (*Sal*) times higher respectively than the threshold of 1,000 mg kg^{-1} given by Italian legislation for industrial sites (Legislative Decree 152/2006). In the case of Zn, the data showed that the mining activities at *Ava* did not result in an anomalous soil accumulation of Zn, whereas they did at *Cap* and *Sal* where they were 15.8 and 5.3 times higher respectively than the threshold of 1,500 mg kg^{-1} given by Italian legislation for industrial sites (Legislative Decree 152/2006) (Table 3.3). Finally, a very high average concentration of Tl (521 mg kg^{-1}) was measured in the

Table 3.3 – Mean, standard error (se) and range values of total concentrations of Cu, Pb, Tl, and Zn in the collected soils.

Site	Cu (mg kg^{-1})		Pb (mg kg^{-1})		Tl (mg kg^{-1})		Zn (mg kg^{-1})	
	Mean (se)	Range	Mean (se)	Range	Mean (se)	Range	Mean (se)	Range
Ava	3,635 (1,045)	267−14,673	3,912 (1,107)	281−16,117	28.8 (5.9)	0.8−93.3	380 (87.2)	78.5−1,227
Cap	18.8 (6.2)	1.3−90.6	7,740 (2,349)	1,156−39,230	521 (88.7)	238−1,411	23,722 (3,494)	10,194−53,418
Cem	2.2 (0.2)	0.8−3.2	0.3 (0.0)	0.3−0.3	3.5 (0.7)	0.4−9.0	25.3 (2.4)	15.1−35.2
Cod	15.9 (1.0)	5.3−17.9	39.9 (3.1)	12.5−51.0	2.9 (0.5)	0.4−6.3	109 (5.7)	65.5−134
Cor	18.3 (0.3)	15.1−20.6	17.8 (0.4)	11.9−19.5	0.4 (0.0)	0.4−0.4	48.8 (0.8)	37.4−51.2
Cra	10.9 (0.8)	7.3−14.7	20.9 (0.4)	18.8−23.3	0.4 (0.0)	0.4−0.4	92.8 (2.1)	82.4−102
Lus	0.6 (0.1)	0.2−1.6	10.2 (1.1)	4.8−17.3	0.4 (0.0)	0.4−0.4	14.0 (0.8)	9.5−19.2
Mon	10.2 (1.1)	2.9−17.9	48.3 (5.6)	13.7−86.1	0.4 (0.0)	0.4−0.5	115 (9.3)	56.0−178
Pic	0.2 (0.0)	0.2−0.2	0.3 (0.0)	0.3−0.3	1.4 (0.4)	0.4−6.0	18.8 (2.4)	4.6−36.2
Pma	0.6 (0.1)	0.2−1.6	6.6 (0.5)	2.8−13.2	0.5 (0.0)	0.4−0.5	21.7 (0.7)	16.7−27.6
Pmc	41.9 (0.2)	40.4−42.6	94.2 (0.3)	91.4−96.7	1.3 (0.2)	0.4−2.7	156 (0.7)	151.0−161
Rib	7.4 (0.2)	6.2−8.9	43.2 (0.1)	37.7−48.1	0.4 (0.0)	0.4−0.4	49.4 (0.8)	43.2−54.8
Rmi	5.0 (0.3)	3.3−7.2	14.1 (1.2)	0.3−20.7	0.7 (0.2)	0.4−3.4	48.9 (1.9)	35.4−66.5
Sal	6.4 (1.3)	0.2−13.2	2,140 (446)	594−6,355	26.4 (7.4)	0.4−118	8,003 (1,133)	4,000−16,975
Vas	0.2 (0.0)	0.2−0.2	1.3 (0.2)	0.3−3.3	0.4 (0.0)	0.4−0.5	11.6 (0.4)	9.2−15.7
	***(†)		***		***		***	

(†) Kruskal–Wallis test (p=0.001)

Cap samples. Thallium occurs as a trace element in several minerals that used to be extracted from this site and was therefore discharged into the mine tailings. At the other mining sites the values were significantly lower, although they were also anomalous with respect to the threshold of 10 mg kg^{-1} given by Italian legislation for industrial sites (Legislative Decree 152/2006) (Table 3.3).

Spearman's correlation coefficients between the concentration of Cu, Pb, Tl and Zn in soils and in plant fractions (roots and shoots) are given in Table 3.4. A positive correlation for Cu, Pb, Tl and Zn soil/root concentration was verified; the same evidence was found in root/shoot and soil/shoot.

Figure 3.2 and 3.3 report shoot-to-soil and shoot-to-root concentrations of Cu, Pb, Tl and Zn, respectively. The open symbols indicate all plant specimens of *Biscutella laevigata* subsp. *laevigata* from non-metallicolous populations. The aforementioned are indicated by different symbols in order to be able to distinguish their origin and behavior in relation to different metals. An evaluation of the magnitude of the metal accumulation in the plant shoots is provided by means of broken lines indicating the hyperaccumulation threshold limits for Cu, Pb and Zn (Baker and Brooks, 1989), and for Tl (Krämer, 2010).

In terms of values, the Cu shoot concentrations in *Ava* specimens ranged from 11.5 mg kg^{-1} up to 212 mg kg^{-1} and in *Ava* and *Cap* specimens the Pb shoot concentrations ranged from 7.2 mg kg^{-1} up to 541 mg kg^{-1} and from 9.3 mg kg^{-1} up to 351 mg kg^{-1} respectively (Figure 3.2 and 3.3). The shoot Zn concentrations in *Cap* and *Sal* specimens ranged from 268 mg kg^{-1} up to 3,669 mg kg^{-1} and from 133 mg kg^{-1} up to 1,182 mg kg^{-1}, respectively (Figure 3.2 and 3.3).

The most interesting results concerned Tl. *B. laevigata* subsp. *laevigata* showed strong Tl tolerance and hyperaccumulation of this element. The Tl concentrations recorded in the shoots of the specimens collected at *Cap* ranged from 247 mg kg^{-1} up to 32,661 mg kg^{-1} (Figure 3.2 and 3.3); the Tl concentration in 14 out of 16 specimens exceeded 1,000 mg kg^{-1}, which is the threshold of hyperaccumulation updated from 500 mg kg^{-1} after Krämer (2010).

One-way Analysis of Covariance (ANCOVA) on rank-transformed data for edaphic type showed that for each metal, the covariates metal concentration in soil and in roots were significant except for the covariate Pb concentration in soil (Table 3.5). Furthermore, edaphic type for Tl was a significant factor affecting shoot metal concentrations with both the covariates, i.e. metal concentration in soil and in roots, thus indicating different slopes for metallicolous and non-metallicolous populations

Table 3.4 – Spearman's correlation coefficient (r) between Cu, Pb, Tl and Zn concentrations in soil, roots and shoots calculated for specimens of *Biscutella laevigata* subsp. *laevigata* collected from 15 different populations in N-E Italy (n=292).

	Cu	Pb	Tl	Zn
Soil/Roots	0.159^{*}	0.388^{***}	0.314^{***}	0.254^{***}
Roots/Shoots	0.600^{***}	0.697^{***}	0.651^{***}	0.688^{***}
Soil/Shoots	0.121^{*}	0.399^{***}	0.431^{***}	0.282^{***}

^{ns} Not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$

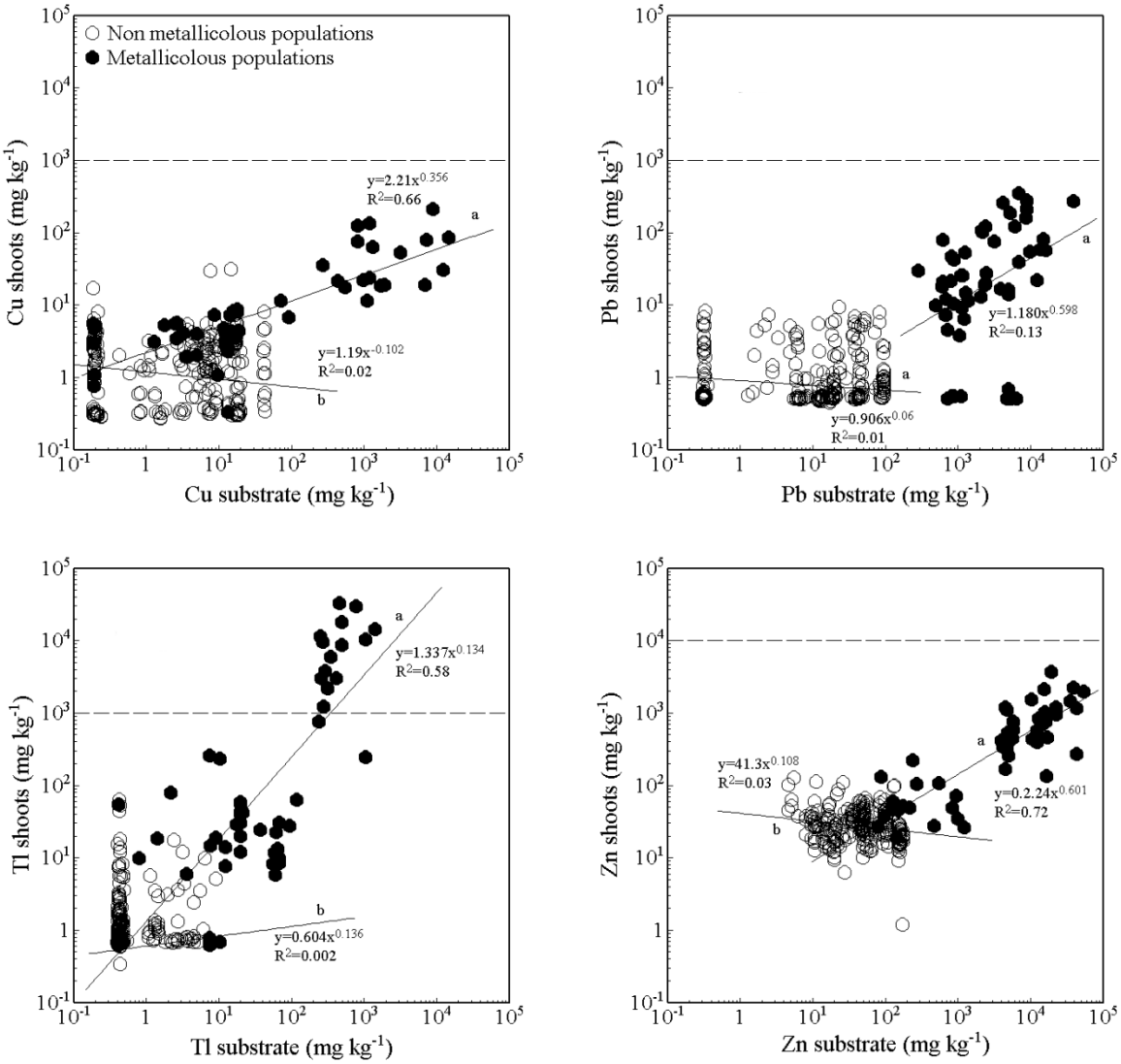

Figure 3.2 − Relationships between concentrations of Cu, Pb, Tl and Zn in soils and in the shoots of *Biscutella laevigata* subsp. *laevigata* specimens collected from metallicolous and non-metallicolous populations. Broken lines represent the metal hyperaccumulation thresholds for Cu, Pb and Zn (Baker and Brooks, 1989) and Tl (Krämer, 2010). The regression lines on power function and their coefficients are provided separately for edaphic types (metallicolous and non-metallicolous populations) for each metal. Shared letters designate regression equations having slopes that are not significantly different according to non-parametric Analysis of Covariance (ANCOVA, $p<0.05$).

in shoot metal accumulation. Edaphic type was also a significant factor for Cu and Zn in affecting shoot metal concentrations but only with the covariate metal concentration in soil. Regarding Pb the edaphic type was not significantly different either with covariate metal concentration in soil or covariate metal concentration in roots. Non-parametric ANCOVA testing the effect of different populations within metallicolous edaphic type separately for Cu, Pb, Tl and Zn shows that the populations were significantly different with both the covariates only for Tl, i.e. metal concentration in soil and in roots. On the

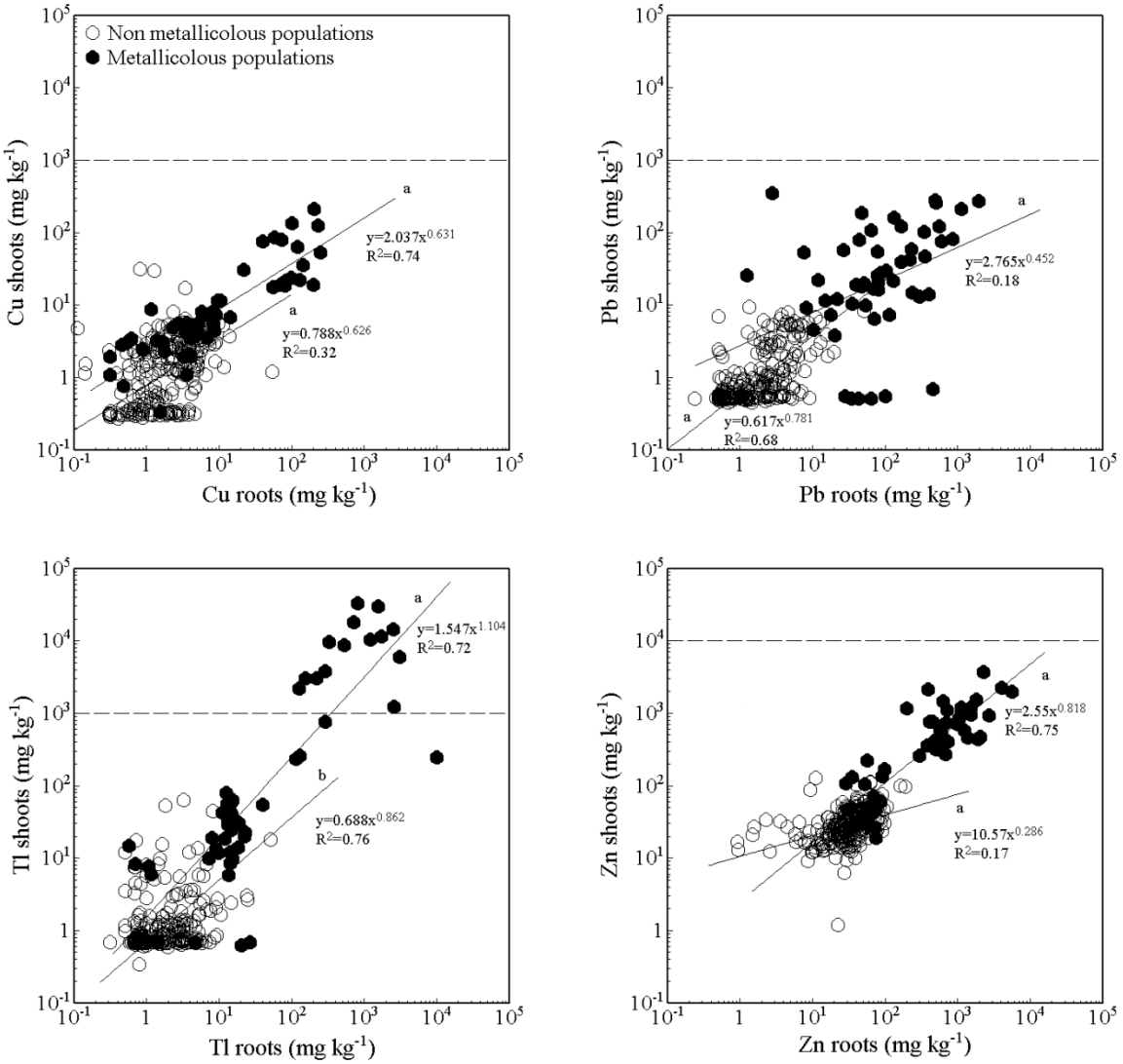

Figure 3.3 − Relationships between concentrations of Cu, Pb, Tl and Zn in the roots and in the shoots of *Biscutella laevigata* subsp. *laevigata* specimens collected from metallicolous and non-metallicolous populations. Broken lines represent the metal hyperaccumulation thresholds for Cu, Pb and Zn (Baker and Brooks, 1989) and Tl (Krämer, 2010). The regression lines on power function and their coefficients are provided separately for edaphic types (metallicolous and non-metallicolous populations) for each metal. Shared letters designate regression equations having slopes that are not significantly different according to non-parametric Analysis of Covariance (ANCOVA, $p<0.05$).

Table 3.5 – Non parametric analysis of covariance (ANCOVA, Conover and Iman, 1982) testing separately for Cu, Pb, Tl and Zn the effect (a) of edaphic types (metallicolous and non-metallicolous) or (b) of different populations within metallicolous edaphic type and ranked soil or roots metal concentrations as covariates on ranked shoots metal concentrations in *Biscutella laevigata* subsp. *laevigata*. Statistics included sum of squared deviations (SS), F statistics and probability (*p*).

(a)	Rank of shoot Cu			Rank of shoot Pb			Rank of shoot Tl			Rank of shoot Zn		
Source	SS	F	*p*	SS	F	*p*	SS	F	*p*	SS	F	*p*
Intercepts among edaphic types	6761.0	1.224	0.269	1761.1	0.343	0.558	17285.6	4.484	0.035	77193.2	15.812	<0.001
Rank of metal in soil(†)	21959.1	3.976	0.047	295.5	0.058	0.810	76728.5	19.904	<0.001	112130.4	22.969	<0.001
Slopes among edaphic types	82409.7	14.920	<0.001	17.8	0.003	0.953	62838.4	16.301	<0.001	144447.3	29.588	<0.001
Error	1629445.3			1420818.4			990734.3			1445047.5		
Intercepts among edaphic types	29756.9	6.956	0.009	4630.9	1.286	0.258	4174.2	1.640	0.202	1586.3	0.419	0.518
Rank of metal in root	313896.1	73.378	<0.001	171392.1	47.602	<0.001	205170.0	80.607	<0.001	339428.2	89.620	<0.001
Slopes among edaphic types	1558.7	0.364	0.547	9791.4	2.719	0.100	18209.3	7.154	0.008	11145.4	2.943	0.087
Error	1227734.0			946929.7			638872.3			1098345.4		

(b)	Rank of shoot Cu			Rank of shoot Pb			Rank of shoot Tl			Rank of shoot Zn		
Source	SS	F	*p*	SS	F	*p*	SS	F	*p*	SS	F	*p*
Intercepts among ME(‡)	519.9	2.919	0.063	288.4	1.334	0.273	865.5	8.756	0.001	867.8	4.602	0.014
Rank of metal in soil	234.0	2.628	0.111	410.4	3.797	0.057	252.5	5.109	0.029	78.7	0.835	0.365
Slopes among ME	1114.1	6.256	0.004	1239.3	5.732	0.006	478.4	4.840	0.013	137.9	0.731	0.486
Error	4541.1			5404.8			1976.8			4997.5		
Intercepts among ME	196.0	1.573	0.217	749.6	3.755	0.031	466.5	5.544	0.007	815.5	4.955	0.011
Rank of metal in root	789.8	12.679	0.001	1508.0	15.108	<0.001	297.4	7.069	0.011	313.6	3.810	0.057
Slopes among ME	47.8	0.383	0.683	186.6	0.935	0.400	322.4	3.831	0.030	263.5	1.601	0.212
Error	3114.7			4591.6			1683.1			3949.8		

(†) The metal is the same as in the corresponding rank of shoot metal; (‡) (ME)=Metallicolous populations

contrary, the metallicolous populations did not differ for Zn, while for Cu and Pb only in the case with the covariate metal concentration in soil (Figure 3.2 and 3.3, Table 3.5).

A post hoc analysis showed that in the within metallicolous adjusted mean ranks of shoot metal concentrations, for both covariates, they were significantly greater in *Cap* than those from *Ava* and *Sal* regarding Tl ($p<0.05$). Concerning Cu and Pb, the adjusted mean rank of shoot metal from *Sal* with the covariate metal concentration in soil was the lowest ($p<0.05$) while *Ava* and *Cap* did not differ significantly.

3.4.2. Thallium tolerance test

The root elongations recorded in plants exposed to increasing concentrations of Tl were used to calculate the Index of Tolerance (IT) (Figure 3.4). As expected, a statistically significant reduction of IT ($p<0.001$) was recorded in response to the increase of Tl concentration in nutrient solution. Estimated from the root growth response, *Cap* plants exhibited a significantly higher Tl tolerance than *Cod* plants ($p<0.001$) (Figure 3.4). In *Cap* plants, there was no significant decrease in root growth below 10 mg L^{-1} ($p<0.001$). On the contrary, in *Cod* plants, all concentrations produced a significant decrease ($p<0.001$).

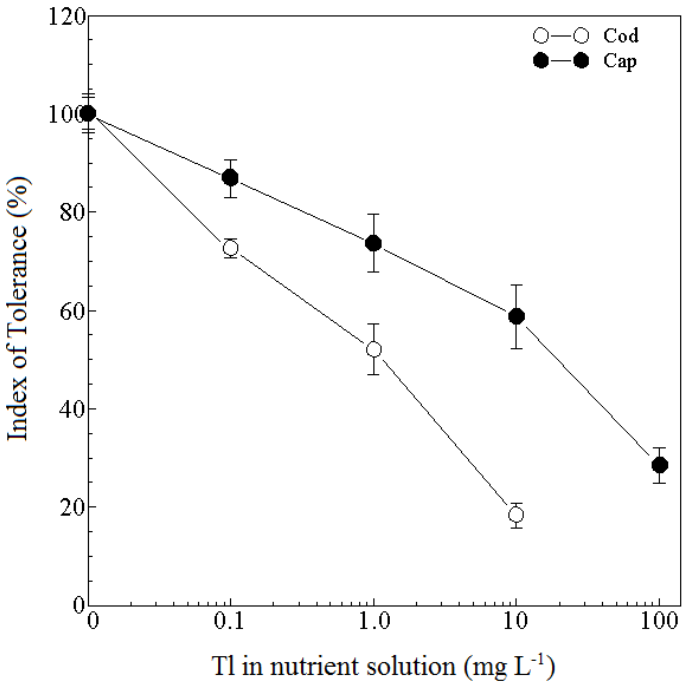

Figure 3.4 – Mean Index of Tolerance (IT) (Schat and ten Bookum 1992) with standard error bars based on root elongation data of two *Biscutella laevigata* subsp. *laevigata* populations (*Cap*, metallicolous population; *Cod*, non-metallicolous population) exposed to various concentrations of $TlNO_3$ for 5 days.

The Tl concentrations in roots and shoots of both populations increased with increasing Tl exposure (data not shown). Root and shoot Tl concentrations were significantly lower in *Cod* as compared to *Cap* ($p<0.001$). Tl concentrations in shoots were significantly higher than in roots in *Cap* ($p<0.001$) while there were no significant differences between shoot and root in *Cod*. *Cap* population reached the highest concentrations at 100 mg L^{-1} ranging from 25,280 mg kg^{-1} to 28,740 mg kg^{-1} and from 12,180 mg kg^{-1} to 16,100 mg kg^{-1} for shoots and roots respectively.

3.4.3. AFLP and genetic analysis

The two AFLP primer combinations produced 59 scoreable peaks: 35 and 24 with the Pst55/Mse47 and Pst55/Mse62 primer combinations, respectively. Control replicates proved that the AFLP data were highly reliable (repeatability 98.6%). The samples for AFLP analysis were less than those for plant analysis because of DNA degradation in some samples.

The Nei and Li (1979) neighbor-joining tree distance of all individuals is shown in Figure 3.5. Only one cluster of possible statistical significance (bootstrap values ≥ 50)

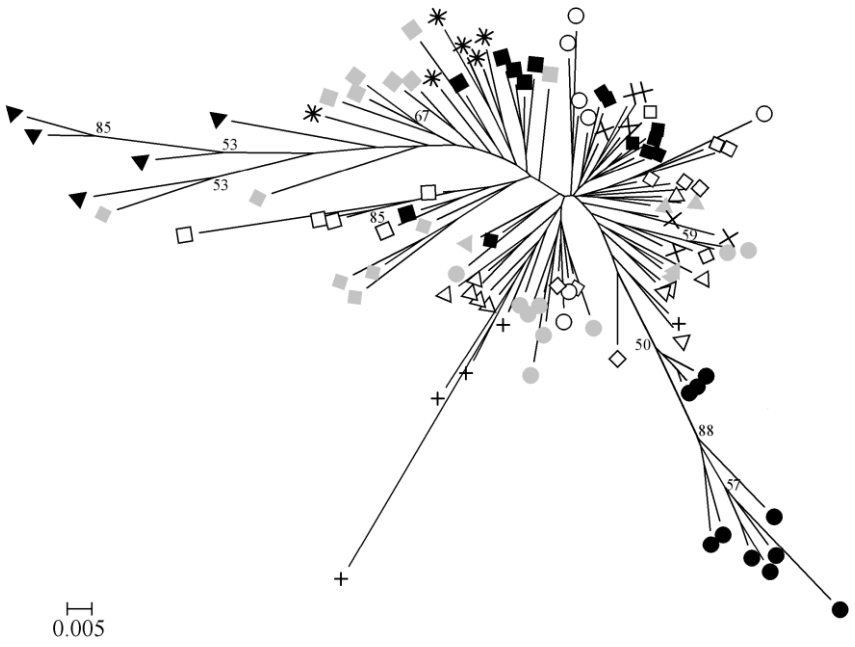

Figure 3.5 – Unrooted neighbor-joining tree of Nei and Li (1979) distances based on Amplified Fragment Length Polymorphism (AFLP) markers describing genetic relationships among 106 specimens of *Biscutella laevigata* subsp. *laevigata* detected in the 15 studied populations: *Ava* (star), *Cap* (black circle), *Cem* (gray circle), *Cod* (white circle), *Cor* (cross mark), *Cra* (black square), *Lus* (gray square), *Mon* (white square), *Pic* (plus sign), *Pma* (black diamond), *Pmc* (gray diamond), *Rib* (white diamond), *Rmi* (black triangle), *Sal* (gray triangle), *Vas* (white triangle). Only bootstrap values greater than 50% are indicated.

corresponding to *Cap* population was detected. All the other populations result as genetically highly related with no evidence of other population specific clusters.

The evidence of unresolved clusters in the neighbor-joining tree, except that of *Cap*, was well supported by Analysis of Molecular Variance (AMOVA) results (Table 3.6). The analysis pointed out no significant difference between edaphic types. Genetic variation within populations greatly exceeded variation among populations (67.6% versus 32.4%, $p<0.001$). Similar results were obtained grouping the populations according to edaphic types even if in the metallicolous populations the differences among populations increased to 44.3% ($p<0.001$). Closer examination , including Φ_{ST} and probabilities of population structure using AMOVA is presented in Table 3.7. All the pair-wised populations resulted as significantly different (Table 3.7).

The relationships tested between the Φ_{ST} estimator of genetic differentiation and geographical distances were not significant, which indicated a lack of isolation by distance.

3.5. Discussion

The results of this study were compared with previous findings (Table 3.8). The early results of investigation on Tl accumulation were reported by LaCoste *et al.* (1999) and Leblanc (1999). Other data on Tl accumulation in *Biscutella laevigata* were provided by Anderson (1999) and Wierzbicka *et al.* (2004). In particular Leblanc *et al.* (1999) reported a maximum Tl concentration of 495 mg kg^{-1} in leaves of specimens sampled at Gailitz, Austria. Unfortunately, no information was provided about the sites from which the plants were collected. However, note that Gailitz is the german name of the Slizza creek, which flows from the mining area of Cave del Predil northwards to Austria. it is therefore likely that those plants were collected from Austrian territory not very far from Cave del Predil (*Cap*, in this work) (Table 3.8).

Rascio (1977) reported Zn accumulation data by *B. laevigata* from Cave del Predil which are in accordance with ours. Another paper by Wenzel and Jockwer (1999) provided data on metal accumulation by *B. laevigata* from metal-rich sites in the Austrian Alps. The most southerly of those sites lies in the Gailitz valley.

In absolute terms, the values of Tl measured in our specimens of *B. laevigata* subsp. *laevigata* collected at Cave del Predil (*Cap*) were very high. In 14 out of 16 specimens the shoot Tl concentration was above the threshold for hyperaccumulation (Figure 3.2 and

Table 3.6 – Analysis of Molecular Variance (AMOVA) of *Biscutella laevigata* subsp. *laevigata* from 15 populations. We considered (a) all the populations (Φ_{ST}), (b) hierarchical analysis of variance among populations within edaphic types (metallicolous vs. non-metallicolous, Φ_{SC}) and between edaphic types (Φ_{CT}) and separate analyses within metallicolous (c) and non-metallicolous (d) populations. Statistics included degrees of freedom (df), sum of squared deviations (SS), estimation of variance components (variance), percentage of total variation contributed by each component (% total), Φ statistics and probability of obtaining a more extreme component estimate by chance alone (*p*). Levels of significance were based on 1023 iteration steps.

Source of variation	df	SS	variance	% total	Φ statistics	*p*
(a) *Whole data set*						
Among populations	14	424.72	3.32	32.4	Φ_{ST}=0.324	<0.001
Among individuals within populations	91	632.56	6.95	67.6		
Total	105	1057.28	10.28			
(b) *Edaphic types*						
Between groups	1	34.75	0.08	0.8	Φ_{CT}=0.007	ns(†)
Among populations within groups	13	389.97	3.30	31.9	Φ_{SC}=0.322	<0.001
Among individuals within populations	91	632.56	6.95	67.3	Φ_{ST}=0.327	<0.001
Total	105	1057.28	10.33			
(c) *Metallicolous populations*						
Among populations within group	2	76.99	4.86	44.3	Φ_{ST}=0.443	<0.001
Among individuals within populations	18	109.87	6.10	55.7		
Total	20	186.86	10.96			
(d*) Non-metallicolous populations*						
Among populations within group	11	304.48	3.10	30.3	Φ_{ST}=0.303	<0.001
Among individuals within populations	68	485.19	7.14	69.7		
Total	79	789.67	10.24			

(†) Not significant (α value≥0.05)

3.3). The highest value of Tl concentration recorded in the shoots of a specimen (32,661 mg kg^{-1}) is, to our knowledge, the highest ever recorded in a higher plant.

Most plants grown in uncontaminated soils have Tl levels in the range 0.02–0.04 mg kg^{-1}, values of 2–7 mg kg^{-1} are considered anomalous (Adriano, 2001). The first data on Tl accumulation were provided by Leblanc *et al.* (1999). Only *B. laevigata*, *Iberis intermedia*, *Silene vulgaris* and *S. latifolia* found in the Les Malines Mining District (France) have so far been found to be able to hyperaccumulate Tl (Anderson *et al.*, 1999, Escarré *et al.*, 2011). However, data from Anderson *et al.* (1999), Leblanc *et al.* (1999) and Escarré *et al.* (2011) did not provide any test for Tl accumulation corrected for the soil Tl concentration, or a Tl tolerance test. Therefore, the Tl tolerance found in *Cap*

Table 3.7 – Distance matrix Φ_{ST} between populations of *Biscutella laevigata* subsp. *laevigata*. Lower matrix – Φ_{ST} defined as the proportion of the total phenotypic variance that is partitioned between two pair wised populations. Upper matrix – probability, defined as the probability that random Φ_{ST} distance > observed Φ_{ST} distance. Levels of significance were based on 1023 iteration steps.

Population	*Ava*	*Cap*	*Cem*	*Cod*	*Cor*	*Cra*	*Lus*	*Mon*	*Pic*	*Pma*	*Pmc*	*Rib*	*Rmi*	*Sal*	*Vas*
Ava		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.01	<0.001	<0.01	<0.001	<0.01	<0.01	<0.001
Cap	0.489		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Cem	0.246	0.394		<0.01	<0.001	<0.01	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001
Cod	0.296	0.443	0.104		<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001
Cor	0.289	0.529	0.208	0.156		<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001
Cra	0.226	0.453	0.176	0.257	0.308		<0.01	<0.01	<0.01	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001
Lus	0.198	0.410	0.299	0.315	0.361	0.200		<0.01	<0.001	<0 .001	<0.001	<0.001	<0.001	<0.001	<0.001
Mon	0.335	0.471	0.300	0.305	0.448	0.292	0.304		<0.01	<0.001	<0.001	<0.001	<0.05	<0.01	<0.001
Pic	0.295	0.338	0.137	0.216	0.307	0.249	0.253	0.332		<0.001	<0.001	<0.001	<0.01	<0.05	<0.001
Pma	0.385	0.617	0.316	0.200	0.202	0.430	0.425	0.486	0.453		<0.001	<0.001	<0.001	<0.01	<0.001
Pmc	0.240	0.321	0.300	0.297	0.360	0.263	0.156	0.314	0.205	0.429		<0.001	<0.01	<0.001	<0.001
Rib	0.320	0.434	0.111	0.075	0.182	0.250	0.321	0.333	0.179	0.255	0.300		<0.001	<0.001	<0.001
Rmi	0.415	0.435	0.468	0.483	0.568	0.495	0.357	0.4189	0.399	0.614	0.272	0.509		<0.05	<0.001
Sal	0.307	0.464	0.213	0.114	0.169	0.308	0.317	0.397	0.203	0.220	0.280	0.126	0.524		<0.01
Vas	0.296	0.4216	0.190	0.150	0.274	0.276	0.260	0.328	0.150	0.297	0.271	0.160	0.459	0.122	

Table 3.8 – Range of concentrations of Cu, Pb, Tl and Zn observed in specimens of *Biscutella laevigata* subsp. *laevigata* found in literature and collected in the Monte Avanza (*Ava*), Cave del Predil (*Cap*) and Salafossa (*Sal*) mining sites.

Location	n[(†)]	Cu (mg kg^{-1})	Pb (mg kg^{-1})	Tl (mg kg^{-1})	Zn (mg kg^{-1})	Reference
Les Aviniéres, France (L) [(‡)]	3			244–308		Anderson *et al.*, 1999
Les Aviniéres, France (W)	34			20–15,199		LaCoste *et al.*, 1999
Tuscany, Italy (W)	15			<1–2		LaCoste *et al.*, 1999
Gailitz, Austria (L)	2			295–495		LeblanC *et al.*, 1999
Arnoldstein, Austria (S)	1		1,090		4,870	Wenzel and Jockwer, 1999
Boleslaw, Poland (S)	5			0.21		Wierbicka *et al.*, 2004
Les Malines, France (A)	10		[(¶)]	0–250	[(¶)]	Escarré *et al.*, 2010
Monte Avanza (*Ava*), Italy (R)	18	10.2–252	7.70–218	9.88–22.1	28.9–85.9	this paper
Monte Avanza (*Ava*), Italy (S)	18	11.5–212	7.20–184	5.73–78.1	18.6–221	this paper
Cave del Predil (*Cap*), Italy (R)	>10				3,600	Rascio, 1977
Cave del Predil (*Cap*), Italy (S)	>10				900–3,600	Rascio, 1977
Cave del Predil (*Cap*), Italy (R)	16	1.4–14	2.76–1,939	126–9,985	197–5,692	this paper
Cave del Predil (*Cap*), Italy (S)	16	n.d.[(§)]–11.4	13.1–351	247–32,661	268–3,669	this paper
Salafossa (*Sal*), Italy (R)	20	0.31–8.47	0.53–460	0.57–132	91.8–1,388	this paper
Salafossa (*Sal*), Italy (S)	20	0.33–5.71	0.51–46.5	0.62–255	133–1,182	this paper

[(†)] Specimens; [(‡)], (L)=leaves, (W)=whole plant, (S)=shoots, (A)=aerial parts, (R)=roots; [(§)] not detectable; [(¶)] see paper

population is a significant novel contribution to evolution and ecology.

Regarding the very high values of Tl concentrations recorded in the shoots of *B. laevigata* subsp. *laevigata*, we excluded that our data could be altered by powder contamination of the leaves of *Cap* specimens. Apart from the standardized laboratory protocols, this problem was carefully considered, leading to the following conclusions: (i) mining activities in the *Cap* area ceased 20 years ago and no human activities currently cause any kind of disturbance on vegetation; (ii) *B. laevigata* subsp. *laevigata* have glabrous leaves and smooth leaf surface; (iii) if the plant samples had been contaminated by dust, abnormal concentrations would have been detected for all metals, but this did not happen; (iv) we tested the dataset for the presence of outliers and some records were erased; (v) a thallium tolerance test was conducted in controlled conditions which also reported high Tl concentration in shoots. Taking these elements into account, we conclude that our data − although having a certain variability − did not suffer from any form of uncontrolled disturbance and therefore they demonstrate the capacity of the plant to accumulate this element.

Novel data with regard to Cu accumulation in *B. laevigata* subsp. *laevigata* are reported in this paper. Copper is an essential nutrient for plants but excessive Cu is highly phytotoxic. In general, Cu phytotoxicity has been associated with 60–125 mg kg^{-1} total soil Cu (Ross, 1994); whereas 20–100 mg kg^{-1} of Cu is considered toxic in most crops (Adriano, 2001).

Ava population specimens of *B. laevigata* subsp. *laevigata* contained Cu concentrations that were higher than those in the other populations. In the specimens belonging to the other populations, the average concentrations of Cu in the roots and shoots were 3.45±0.38 and 3.04±0.35 (mg kg^{-1}, mean ± se), respectively. In the *Ava* population the corresponding Cu concentrations were 109±17 and 57±12 (mg kg^{-1}, mean ± se), respectively. The toxicity threshold of Cu (100 mg kg^{-1}) was exceeded in the roots of eight specimens; the highest Cu concentrations found in roots and shoots were 252 and 212 mg kg^{-1}, respectively.

On the other hand the Zn and Pb concentrations in metallicolous populations were not as high as reported in Wenzel and Jockwer (1999) (Table 3.8).

As expected for a pseudometallophyte, *B. laevigata* subsp. *laevigata* responded positively to the soil concentrations of several metals, first accumulating the metals in the roots and then translocating them to the shoots (Figure 3.2 and 3.3). It is evident that the plants growing on metal-rich substrates tend to have higher metal concentrations in their

shoots. Therefore, since the soil and the root metal concentrations affect shoot metal concentrations, it was necessary to adjust population means for variation among samples in soil and root metal concentrations. The non-parametric Analysis of Covariance (ANCOVA) also indicated that, in the case of Cu, Tl and Zn, metallicolous populations resulted as clearly separated from non-metallicolous populations in terms of shoot metal concentration. Significantly different slopes of Tl accumulation in shoots of *Cap* could be interpreted as a differential behavior of this population for metal accumulation.

A clear pattern of edaphic distribution was not found in the case of Pb. There are apparently some individuals growing on Pb-rich soils that hardly accumulate any Pb in their shoots above background levels. This degree of metal exclusion from the shoot is remarkable, and quite distinct from the pattern shown for Cu, Tl and Zn. These individuals belong to *Sal* population which seems to have a different behavior compared to the other metallicolous populations, probably reflecting a different adaptation.

Thallium tolerance and hyperaccumulation is a rare phenomenon. Metal tolerance refers to specific individuals or populations (genotypes) of a species that are able to withstand greater amounts of toxicity than their immediate relatives on normal soil (Antonovics *et al.*, 1971). It is generally accepted that in metalliferous habitats the evolution of metal tolerance and genetic adaptation to extreme environmental conditions can occur rapidly. However, it is still necessary to test the phenotypical differentiation for metal tolerance and hyperaccumulation traits for each species (or each geographical region within each species) between the metallicolous population in which local adaptation is expected and non-metallicolous populations. Therefore, proving this particularly phenotypical differentiation/adaptation (different tolerance and/or different accumulation behavior) as suggested by non-parametric ANCOVA required a thallium tolerance test in a controlled environment (Schat and ten Bookum, 1992) with increasing Tl concentrations on *Cap* and one non-metallicolous population represented by *Cod*. Population-specific genotype × thallium concentration interactions were observed. The Index of Tolerance (IT) suggests that Tl tolerance in *Cap* population results from both constitutive and adaptive traits.

Due to the results suggesting a particular adaptation of *Cap* population, a population genetic structure analysis using neutral molecular markers for disentangling selective and demographic history was performed. As this autotetraploid species does not follow Hardy-Weinberg expectations (Parisod and Christin, 2008), the genetic structure of 15 different populations was analyzed using a band-based approach on Amplified Fragment

Length Polymorphism (AFLP) data following Bonin *et al.* (2007).

Our survey revealed a high to moderate level of genetic variation within a population, with an average of 53.6% of loci being polymorphic per population. The existence of considerable variation within each population is also evident from the fact that each individual was characterized by a unique AFLP phenotype. Contrary to the expectation a significantly lower genetic variability of the metallicolous compared to non-metallicolous populations was not detectable. Apparently selection on metalliferous soils in *B. laevigata* subsp. *laevigata* is not perceivable or its effect on genetic variability is balanced by gene flow.

Analysis of Molecular Variance (AMOVA) showed that a higher proportion of the genetic variation resided within populations (67.6%), and a relatively lower degree of genetic variation resulted from differentiation among populations (32.4%). Analysis of Molecular Variance showed little evidence of genetic structure and no genetic differentiation between edaphic types. These results are in agreement with those obtained for other metallophyte species where AMOVA was performed on edaphic type groups (Verkleij *et al.*, 1989; Vekemans *et al.*, 1992; Mengoni *et al.,* 2000; Baumbach and Hellwig, 2003, 2007; Basic *et al.*, 2006).

Moreover none of the relationships tested between genetic and geographical distances were significant, which pointed to a lack of isolation by distance according to Parisod and Besnard (2007).

Interestingly, the neighbor-joining tree with bootstrap analysis does appear to show *Cap* portioning from other populations. It is therefore evident that some differentiation occurred in the local *Cap* population.

3.6. Conclusions

Specimens of *Biscutella laevigata* subsp. *laevigata* were collected from soils with normal or slightly high metal concentrations and from soils with anomalous concentrations, confirming the typical behavior of pseudometallophytes. Most populations of pseudometallophytes grow on non-metalliferous soils. When plants colonize metalliferous soils there is strong selection favoring the metal-tolerant genotypes, causing a rapid increase in the average tolerance of the local populations and leading to the establishment of a metal-tolerant ecotype.

In this paper, we provide remarkable evidence on the metal tolerance of *B. laevigata*

subsp. *laevigata*, especially regarding Tl. Further work is needed to better focus upon this possibility for other elements. It is likely that the metal-polluted environment and the metalliferous native soils exert selective pressure on a local population of *B. laevigata* subsp. *laevigata* in Cave del Predil. This is confirmed by metal concentrations found in the plant tissues, the index of Tl tolerance and cluster analysis.

Acknowledgements

This research was part of the project "Persistent green capping on polluted or metalliferous sites: functional role and reservoir of biodiversity" supported by the Italian Ministry of University and Research (PRIN 2008, N. 2008JZ27T7). Special thanks are due to Roberto Zucchini who provided useful information about ancient mining sites and mineralizations in Friuli Venezia Giulia, and to Massimo Buccheri for providing data from the herbarium collections of the Museo Friulano di Storia Naturale (Udine).

Notes on contributors

Filip Pošćić is doctoral student at University of Udine in Italy. His doctoral research is focused on ecology and genetics of metallophytes; Guido Fellet conducted his doctoral research on phytoremediation of trace elements with metallophytes and agronomic species. He is currently a post-doctoral researcher at University of Udine, Italy; Valentino Casolo is a senior researcher at the Department of Agricultural and Environmental sciences of University of Udine, Italy. He currently conducts research on anthropogenic vegetation traits and plant ecophysiology, especially focused on invasive species; Luca Marchiol is senior researcher at University of Udine, Italy. He currently conducts research on phytotechnologies, metallophytes and restoration ecology; Massimo Vischi is senior scientist at University of Udine, Italy. He currently conducts research on plant biodiversity and DNA barcoding of land plants.

4

Hyperaccumulation of thallium is population-specific and uncorrelated with caesium accumulation in the thallium hyperaccumulator, *Biscutella laevigata*

Filip Pošćić[1], Luca Marchiol[1] and Henk Schat[2]

[1]Dipartimento di Scienze Agrarie e Ambientali, Università degli Studi di Udine, Via delle Scienze 208, I-33100 Udine, Italy; [2]Department of Genetics, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081 HV Amsterdam, The Netherlands

Submitted to New Phytologist

4.1. Abstract

Thallium and caesium tolerance and accumulation were compared in one non-metallicolous and three metallicolous (calamine) populations of the candidate Tl hyperaccumulator species, *Biscutella laevigata*.

Plants were grown hydroponically and exposed to a series of Tl or Cs concentrations. Population tolerance and accumulation were evaluated. Moreover, Tl and Cs accumulation were measured at different K concentrations in the nutrient solution. Seedlings were also grown in Tl contaminated calamine soil.

Estimated from their root growth response, all the calamine populations showed hypertolerance to Tl, although to very different degrees. Cs was much less toxic then Tl, and Cs tolerance was significantly enhanced in the two most Tl-tolerant populations. Foliar Tl hyperaccumulation from hydroponics and soil was apparent in two populations. Tl hyperaccumulation was a high-affinity phenomenon in one of them, but in another one it was only apparent at extremely high Tl exposure levels, and not associated with enhanced root-to-shoot translocation. There was no considerable inter-population variation in Cs accumulation, except that one population showed a relatively low Cs retention in its roots under low exposure.

Tl hyperaccumulation and hypertolerance are population-specific traits in *B. laevigata*. Cs accumulation and tolerance are much less variable and largely uncorrelated with Tl accumulation and tolerance.

Keywords: thallium, caesium, tolerance, hyperaccumulation, translocation, *Biscutella laevigata*

4.2. Introduction

Thallium is a toxic heavy metal, which locally occurs as an environmental contaminant. Tl is more toxic to mammals (Zitko, 1975), or plants (Allus *et al.*, 1988) than Cd, Cu, Hg, Pb, or Zn. Therefore Tl is listed by USEPA as one of the 13 priority pollutants (Keith and Telliard, 1979). Tl toxicity is believed to result from its high affinity towards S and P containing ligands, leading to substitution of K at specific binding sites. This interferes with K-metabolism and, in many cases, with the formation of ATP in respiration (Sager, 1994). Early studies of Siegel and Siegel (1975, 1976a,b) have shown that K is an inhibitor of Tl toxicity, while Cataldo and Wildung (1978) reported that absorption of Tl by plants is under metabolic control, and that K acts as a non-competitive inhibitor of Tl uptake. Günther and Umland (1989) have shown that the major part of Tl exists as free Tl(I) ions in *Brassica napus*. This was also found for *Sinapsis alba* and *Entondon schreberi* (Krasnodębska-Ostręga *et al*., 2008). However, within in the leaves Tl is closely associated with Ca, predominantly localized within the vascular system (Günther and Umland, 1989). Tl can be readily taken up by plants because it is generally present in soil as thermodynamically stable Tl(I), which is chemically similar to K(I) (Nolan *et al*., 2004, Galván-Arzate and Santamaría, 1998).

Caesium is much less toxic than Tl (Bystrzejewska-Piotrowska and Urban, 2003; Hampton *et al.*, 2004). It is mined mostly as pollucite, and its concentrations in normal soils are low and non-toxic (White and Broadley, 2000). Bystrzejewska-Piotrowska and Urban (2003) showed that Cs accumulation in leaves of *Lepidium sativum* negatively affects the water balance, tissue hydration, gas exchange parameters, and biomass productivity. As a late effect, a decrease of photosystem II yield was also observed. Hampton *et al*. (2004) have shown that Cs phytotoxicity in *Arabidopsis thaliana* results from competition between K and Cs for K-binding sites on essential proteins. Only in areas containing Cs-rich pollucite ores, such as those found in southern-eastern Manitoba (Teertstra *et al.*, 1992), might Cs cause environmental toxicity (White and Broadley, 2000).

However, radioisotopes of Cs are produced as fission products in nuclear reactors. Owing to incidents involving nuclear power plants, these isotopes can be released into the environment. Especially ^{137}Cs deserves attention because of its high fission yields, long half-life ($t_{1/2}$=0.2 years), and influence on human health (Dushenkov, 2003). Because of the pronounced radiotoxicity and the diffuse nature of ^{137}Cs pollution, it has been

considered desirable to develop phytoremediator crops that could effectively phytoextract it from the soil within a short period (Dushenkov, 2003).

Similar to Tl, also Cs is a chemical analogue of K, and is therefore relatively easily taken up by plants (White and Broadley, 2000). Kanter *et al*. (2010) recently identified an amino acid substitution close to the K-pore, the cyclic nucleotide gated channel 1 (CNGC1) in the Sorbo accession of *Arabidopsis thaliana*, which is probably responsible for the relatively high Cs^+ accumulation capacity in this accession.

Up to the present, no natural hyperaccumulators of Cs have been reported. However, since both Tl and Cs are chemical analogues of K, it seems not unlikely that Cs hyperacumulators, whenever they exist at all, might be found among the species that have been reported to hyperaccumulate Tl. These species are: *Galium* sp., with 1.7% (w/w) Tl in flowers (Zyka, 1972), *Iberis intermedia*, with 1.3% Tl in mature leaves (Scheckel *et al*., 2004), *Silene latifolia*, with 0.15% Tl in shoots (Escarré *et al.*, 2011), and *Biscutella laevigata*, with 1.5% Tl in whole-plant dry matter (Anderson *et al*., 1999), even up to 3.3% Tl in shoots (Pošćić *et al*., unpublished). Exceptional Tl accumulation has also been reported for *Brassica oleracea* var. *acephala* (Pavlíčková *et al*., 2006), *Lolium perenne* (Al-Attar *et al*., 1988) and *Plantago lanceolata* (Wierzbicka *et al*., 2004), whereas *Brassica napus*, *Dianthus carthusianorum*, *Silene vulgaris*, *Triticum aestivum* and *Zea mays* exhibit variable, but relatively low or negligible levels of Tl accumulation (Tremel *et al*., 1997; Al-Najar *et al*., 2005, Wierzbicka *et al*., 2004, Escarré *et al.*, 2011).

Regarding Tl hyperaccumulation, *B. laevigata* seems to be a very interesting species, because there is evidence of considerable intra-specific variation. *Biscutella laevigata* L. (Brassicaceae) is a European, yellow-flowering hemicryptophyte with a sporophytic self-incompatibility system (Olowokudejo and Heywood, 1984). It is a 'facultative metallophyte', with both non-metallicolous and metallicolous populations, the latter showing population-specific hypertolerances to the metals that are present at toxic concentration in the soil at their population sites, e.g., Pb-Zn mine waste deposits (Wierzbicka and Pielichowska, 2004). The reports on Tl hyperaccumulation in *B. laevigata* are contradictory. The populations from the Pb-Zn mines Les Avinières, near the village of Ganges, South France, and Cave del Predil, Tarvisio, North Italy, are doubtlessly able to hyperaccumulate Tl, with foliar Tl concentrations far in excess of those in the soil, which precludes the possibility of significant contributions of airborne contamination. On the other hand, the population from the Pb-Zn mine waste deposit at Bolesław, Olkusz, Poland, is definitely not hyperaccumulating Tl, in spite of the relatively

high soil Tl concentration at this site (Wierzbicka *et al*., 2004). These field observations clearly suggest that Tl hyperaccumulation capacity is a population-specific rather than a species-wide trait in *B. laevigata*, but experimental confirmation is lacking to date. In addition, it is not clear whether the colonization of metalliferous, calamine soils has lead to Tl hypertolerance in this (or any other) metallophyte, and if so, whether such Tl hypertolerance is confined to Tl hyperaccumulating populations. Therefore, the first aim of this study was to compare the tolerance and accumulation of Tl among different non-metallicolous and metallicolous *B. laevigata* populations under controlled experimental conditions. In addition, we addressed the question of whether Cs tolerance and accumulation is predictable from Tl tolerance and accumulation characteristics.

4.3. Materials and methods

4.3.1. Plant materials and experimental conditions

Seeds of *Biscutella laevigata* were collected from plants growing at the former Pb-Zn mine tailing ponds of Cave del Predil (northern Italy) (*Cap*) and Les Avinières (southern France) (*Lea*), from plants growing at the Pb-Zn smelter waste deposits from Bolesław (southern Poland) (*Bol*) and from non-metalliferous soil in the Tatra Mountains (southern Poland) (*Tat*). A brief description of the collection sites is given in Table 4.1.

Seeds were sown in a garden peat soil (Jongkind, nr. 7, Aalsmeer, Netherlands) and left for three weeks in a growth chamber. Seedlings were then transferred to hydroponic culture, in 1-L polyethylene pots (one plant per pot) containing a modified half-strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.50 mM $MgSO_4$, 20 mM Fe(Na)eEDTA,1 µM KCl, 25 µM H_3BO_3, 2 µM $MnSO_4$, 2 µM $ZnSO_4$, 0.1 µM $CuSO_4$ and 0.1 µM $(NH_4)_6Mo_7O_{24}$ in demineralised water buffered with 2 mM 2-N-morpholino-ethanesulphonic acid (MES), pH 5.5, adjusted with KOH. Nutrient solutions were renewed weekly and plants were grown in a growth chamber (20/15 °C day/night; light intensity 220 µE m^{-2} s^{-1}, 14 h day^{-1}; relative humidity 75%).

4.3.2. Testing Tl and Cs tolerance

After 10 days of pre-culture, plants were exposed to a series of Tl or Cs (0, 4, 16, 32, 64, 128, 256 µM $TlNO_3$ or CsCl) concentrations (10 plants per population per concentration), in a background solution of the same composition as the pre-culture solution. Prior to exposure, roots were stained with active coal powder to facilitate the

Table 4.1 – Description of the sites of collection of *Biscutella laevigata* seeds.

Site	Code	Habitat description	Altitude (m)	Latitude (N)	Longitude (E)	Tl (mg kg^{-1})	References
Bolesław (Olkusz, S Poland)	*Bol*	Pb-Zn calamine waste heaps	313	50° 17' 35"	19° 29' 08"	43±12(†)	Wierzbicka *et al.*, 2004
Cave del Predil (Tarvisio, N Italy)	*Cap*	Former Pb-Zn minin site	901	46° 26' 26"	13° 34' 16"	521±88.7(‡)	Pošćić *et al.*, unpublished
Les Avinières (Ganges, S France)	*Lea*	Former Pb-Zn mining site	175	43° 55' 59"	3° 39' 59"	7.2–115.1(§)	Escarré *et al.*, 2011
Tatra Mountains (S Poland)	*Tat*	Natural mountain site	1,100	49° 15' 12"	19° 55' 54"	-(¶)	

(†) Mean±SD; (‡) Mean±SE; (§) Range of means from different soil Horizons; (¶) Not known.

measurement of root growth (Schat and ten Bookum, 1992). After 5 days of exposure, the length of the longest unstained root segment was measured (Schat and ten Bookum, 1992).

4.3.3. Growth in contaminated soil

Seeds (5 plants per population) were sown directly in 1-L pots with a perforated bottom, filled with contaminated soil containing 118±4.66 (mean ± se) mg kg^{-1} d.w. of Tl from the former Pb-Zn mine tailing ponds of Cave del Predil (southern Italy). Water was added weekly via the dishes under the pots, thus avoiding leaching. After the development of the third leaf, the plants were harvested, the cotyledons were removed, and the roots were carefully washed in demineralised water. Then the plants were dried in a stove (70 °C) and stored until analysis.

4.3.4. Testing the effect of K supply on Tl and Cs accumulation

After 10 days of pre-culture, plants were exposed to 2 and 8 μM $TlNO_3$ or CsCl in a background solution of the same composition as the pre-culture solution, except for the K concentrations, which were set at 1, 3, or 6 mM KNO_3. Ten plants per population per concentration were exposed for 5 days and then analysed.

4.3.5. Determination of Tl and Cs concentrations

Tl and Cs concentrations were determined in roots and shoots (10 plants per population per concentration). Root material was carefully rinsed with ice-cold lead nitrate (5 mM) for 30 min to desorb metals from the root free space and then blotted with paper tissue. Tl and Cs were determined by digesting 20 – 100 mg of oven-dried plant material in 2 ml of a 1 to 4 (v/v) mixture of 37% (v/v) HCl and 65% (v/v) HNO_3 in Teflon cylinders for 7 h at 140 °C, after which the volume was adjusted to 10 ml with demineralised water. Tl and Cs were determined using atomic absorption and flame emission spectrophotometry, respectively (Perkin Elmer 2100; Perkin Elmer Nederland, Nieuwerkerk a/d Yssel, The Netherlands).

4.3.6. Statistics

Statistical analysis was carried out with one-, two- and three-way ANOVA. A posteriori comparison of individual means was performed using Tukey's test. Data were subjected to logarithmic transformation prior to analysis, which effectively homogenized

variances.

4.4. Results

4.4.1. Effects of Tl and Cs on root growth

As estimated from the root growth response, there was a remarkable inter-population variation in Tl tolerance (Figure 4.1). The *Cap* population was the most tolerant, showing significant root growth reduction exclusively at the highest (256 μM) Tl treatment, followed by the *Lea* population. The latter showed significant growth reduction already at the next highest (128 μM) Tl treatment and complete growth inhibition at the highest. The third metallicolous population, *Bol*, was much more sensitive than *Lea* and *Cap*, showing significant growth reduction already at 16 μM Tl, but significantly more tolerant than the non-metallicolous population, which was already significantly inhibited at 4 μM Tl.

In comparison with Tl tolerance, Cs tolerance varied much less among the populations (Figure 4.2). At the highest concentration (256 μM Cs), all the populations maintained considerable root growth rates, which were significantly lower than in the controls only in *Bol* and *Tat* (p<0.05), but not so in *Lea* and *Cap*. When compared at the same concentration, significant differences were only found at the 128-μM Cs treatment, where *Lea* and *Cap* performed slightly, but significantly better than *Bol* and *Tat* (p<0.05).

4.4.2. Tl and Cs accumulation in hydroponics

For Tl accumulation in roots and shoots, we performed the statistical analyses (one-way ANOVA, followed by Tukey's test) first with the data from the lowest Tl treatment (4 μM), since this was the only treatment that allowed at least some root growth in all of the populations. The root Tl concentrations decreased in the order *Cap* > *Lea* > *Bol* > *Tat*, each of the populations being significantly different from all of the others (p<0.05). At 16 μM Tl the same order was found, with *Cap* and *Lea* showing significantly higher root Tl concentrations than *Bol* (*Tat* was not included, because its root systems were dead, or close to dead). At exposure levels higher than 16 μM the root Tl concentration levelled off in *Lea*, but continued to increase sharply in *Cap*, up to the highest exposure level (Figure 4.3).

The Tl concentrations in the shoot showed a pattern very different from that in roots. At the 4-μM exposure level the highest shoot Tl concentration was found in *Lea*, i.e. about four-fold higher than in *Cap*, and about seven-fold higher than in *Bol* and *Tat*. The same

pattern was found at the 16 µM Tl exposure level. At 32 µM Tl the shoot concentration levelled off in *Lea*, but continued to increase with exposure level in *Cap*, resulting in almost equal shoot concentrations at the 128 µM-exposure level (Figure 4.4).

The shoot to root Tl concentration ratios varied strongly and significantly between populations, decreasing in the order *Lea* > *Tat* / *Bol* > *Cap*, where "/" indicates a non-significant difference. The ratio was consistently higher than unity in *Lea*, increasing with the Tl exposure level from 2.5 at 4 µM to 3.5 at 128 µM. In the other populations the ratios were close to unity (*Tat* / *Bol*), or significantly lower than unity in *Cap*, increasing from 0.4 at 4 µM to 0.8 at 256 µM Tl.

The root Cs concentrations were significantly lower in *Cap*, at all the exposure levels,

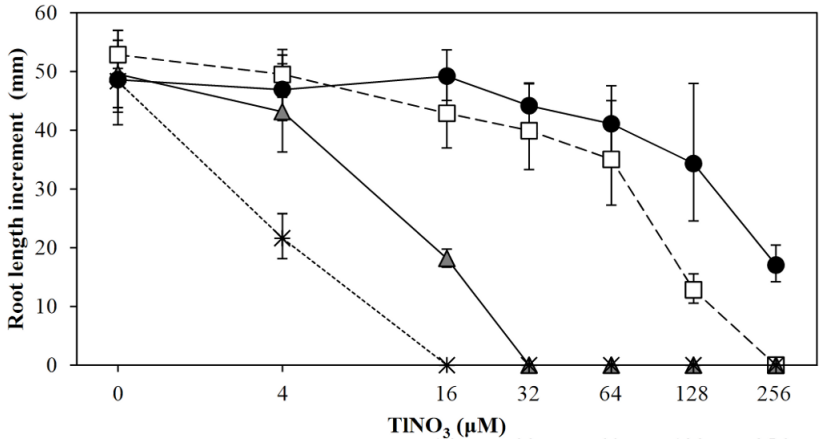

Figure 4.1 – Tl-imposed root growth inhibition (mm) of four populations of *Biscutella laevigata* (triangles, *Bol*; circles, *Cap*; squares, *Lea*; stars, *Tat*) (median±se, n=10) after exposure to increasing Tl concentrations (µM) for 5 days.

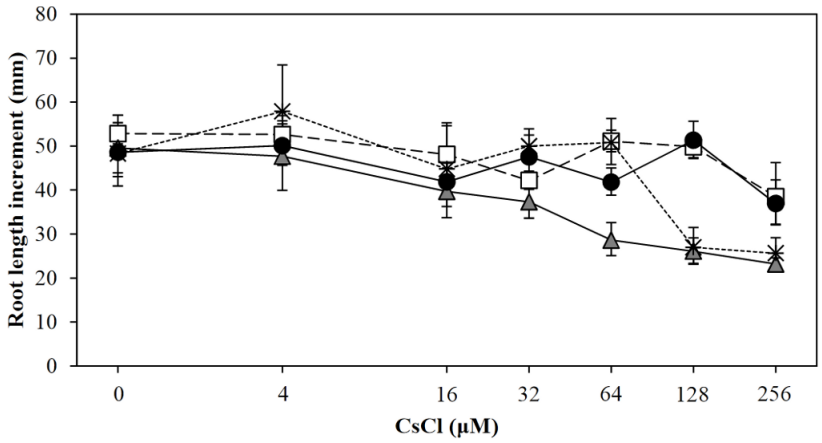

Figure 4.2 – Cs-imposed root growth inhibition (mm) of four populations of *Biscutella laevigata* (triangles, *Bol*; circles, *Cap*; squares, *Lea*; stars, *Tat*) (median±se, n=10) after exposure to increasing Cs concentrations (µM) for 5 days.

than in the other populations, which were not significantly different among each other (Figure 4.5). The shoot Cs concentrations did not significantly vary among the populations (Figure 4.6).

As expected, the Cs shoot to root concentration ratio was significantly higher in *Cap*, decreasing with exposure level from 11.1 at 4 µM to 1.7 at 256 µM, in comparison with the other populations, in which the ratio decreased from 2.0 at 4 µM to 1.4 at 256 µM Cs.

4.4.3. Tl accumulation from mine soil

The populations performed very distinctly in soil from the Cave del Predil mine tailing. The *Tat* plants were stunted and heavily chlorotic, and died before the end of the

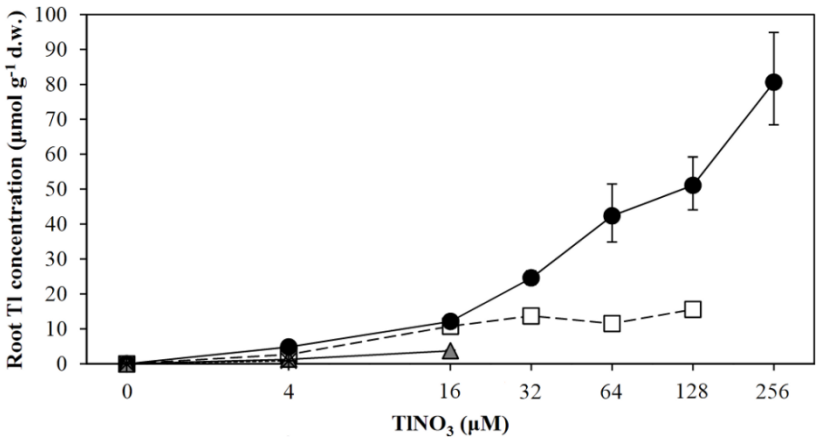

Figure 4.3 – Root Tl concentrations (µmol g^{-1} d.w.) of four populations of *Biscutella laevigata* (triangles, *Bol*; circles, *Cap*; squares, *Lea*; stars, *Tat*) (median±se, n=10) after exposure to increasing Tl concentrations (µM) for 5 days.

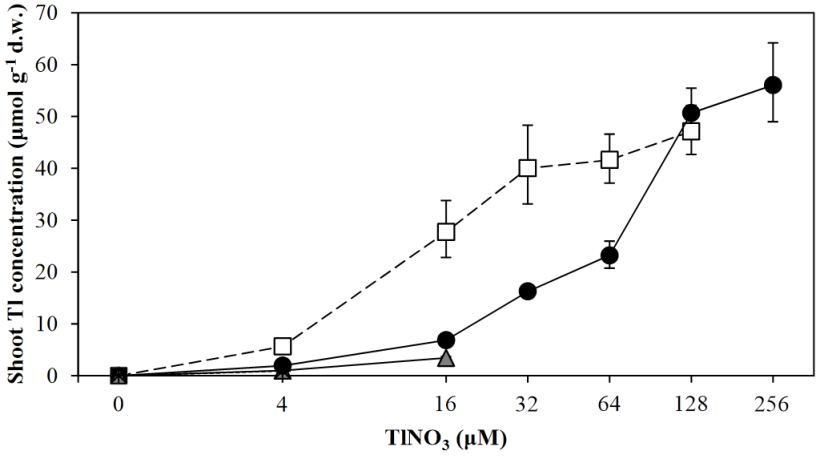

Figure 4.4 – Shoot Tl concentrations (µmol g^{-1} d.w.) of four populations of *Biscutella laevigata* (triangles, *Bol*; circles, *Cap*; squares, *Lea*; stars, *Tat*) (median±se, n=10) after exposure to increasing Tl concentrations (µM) for 5 days.

experiment. Also the *Bol* plants were stunted, but they survived. Only the *Cap* and *Lea* plants developed normally, without displaying toxicity symptoms. The plants from the *Cap* and *Lea* populations showed significantly lower (p<0.01) Tl concentrations in their roots than did the *Bol* plants (Figure 4.7). The shoot Tl concentrations were significantly different among all the populations (p<0.001), decreasing in the order *Lea* > *Cap* > *Bol*. The root to shoot Tl concentration ratios decreased in the order *Lea* > *Cap* > *Bol* (Figure 4.7).

4.4.4. Testing the effect of K on Cs and Tl accumulation

The root and shoot Tl concentrations were significantly affected by the K

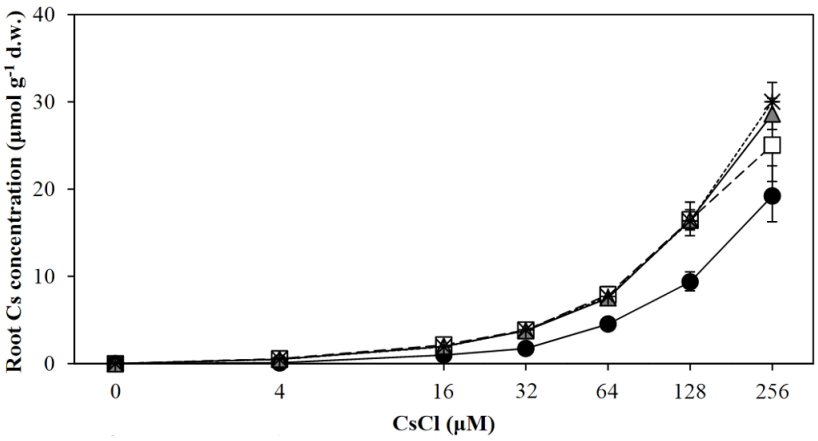

Figure 4.5 – Root Cs concentrations (µmol g^{-1} d.w.) of four populations of *Biscutella laevigata* (triangles, *Bol*; circles, *Cap*; squares, *Lea*; stars, *Tat*) (median±se, n=10) after exposure to increasing Cs concentrations (µM) for 5 days.

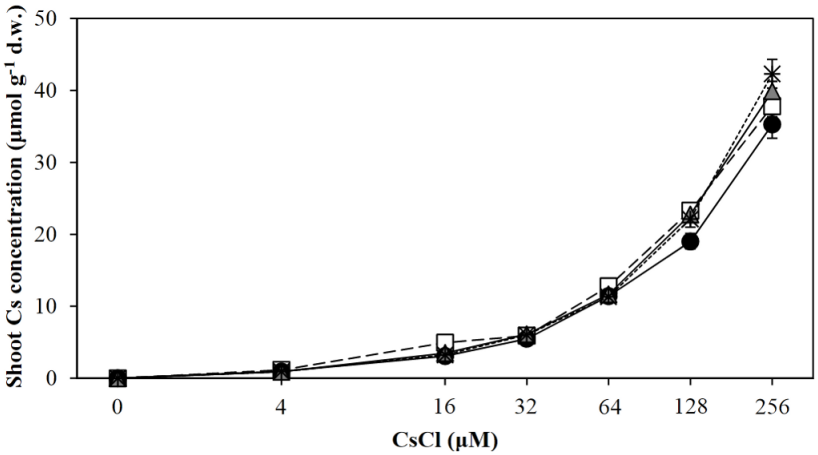

Figure 4.6 – Shoot Cs concentrations (µmol g^{-1} d.w.) of four populations of *Biscutella laevigata* (triangles, *Bol*; circles, *Cap*; squares, *Lea*; stars, *Tat*) (median±se, n=10) after exposure to increasing Cs concentrations (µM) for 5 days

concentration in the nutrient solution (three-way ANOVA, $p<0.001$). However, the population × K interaction was significant too ($p<0.001$), both for shoots and roots, showing that there was inter-population variation in the nature or degree of the K effect (Table 4.2). In particular, the shoot Tl concentrations in the highest K treatment (6 mM) were more strongly decreased (about 50%), in comparison with those in the lowest K treatment (1 mM), in *Bol* and *Lea* (at 8 µM Tl) than they were in *Cap* and *Tat*. The root Tl concentrations were strongly decreased by high K in *Bol* and *Cap*, but not at all or insignificantly in *Lea* and *Tat*. The effect of high K on the shoot to root Tl concentration ratios was also significantly population-specific ($p<0.001$), i.e. increasing in *Cap* (only at 6 mM K), decreasing in *Lea*, and insignificant in *Bol* and *Tat*, suggesting that K affected both Tl uptake and Tl root to shoot translocation in a population-specific way.

The root and shoot concentrations of Cs were also significantly affected by high K supply, albeit much less than the Tl concentrations (Table 4.2). The population × K interaction was insignificant for the shoot Cs concentrations, but highly significant ($p<0.001$) for the root ones, due to the relatively strong effects in *Cap* at 3 and 6 mM K, and in *Lea* at 6 mM K (Table 4.2), with parallel effects on the shoot to root concentration ratios.

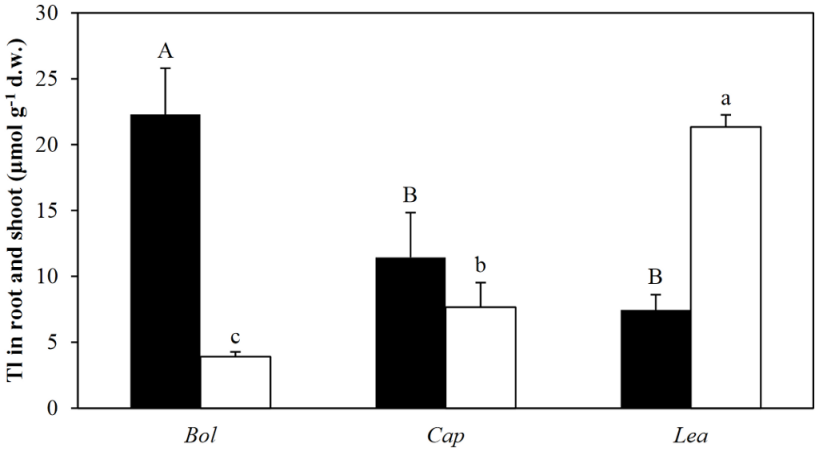

Figure 4.7 – Roots (black columns) and shoots (white columns) Tl concentrations (µmol g^{-1} d.w.) of three *Biscutella laevigata* populations (*Bol*, *Cap*, *Lea*) (mean±se, n=5) after growing in contaminated soil from the former Zn/Pb mine tailing ponds of Cave del Predil (southern Italy). The *Tat* plants were not capable to survive. Different letters indicate significant differences between treatments ($p<0.01$, Tukey's test). Capital letters refer to roots while lowercase letters to shoots.

Table 4.2 – Median±se for (a) Tl or (b) Cs accumulation in shoots or roots at different K and Tl or Cs concentrations in hydroponics in four populations.

(a)		2 µM Tl in solution				8 µM Tl in solution				Tukey's between populations[(†)]	
Plant fraction	K (mM)	*Bol*	*Cap*	*Lea*	*Tat*	*Bol*	*Cap*	*Lea*	*Tat*	2 µM Tl in solution	8 µM Tl in solution
Shoot	1	0.67±0.04	1.40±0.13	12.13±0.49	0.69±0.04	2.56±0.16	5.43±0.47	36.73±5.19	2.10±0.10	*Lea* > *Cap* > *Bol* / *Tat*	*Lea* > *Cap* > *Bol* / *Tat*
	3	0.53±0.03	0.97±0.08	10.80±0.60	0.46±0.04*	1.80±0.25	3.49±0.25*	17.58±2.28*	2.12±0.12	*Lea* > *Cap* > *Bol* / *Tat*	*Lea* > *Cap* > *Bol* / *Tat*
	6	0.38±0.04*	0.87±0.03*	9.76±0.72	0.44±0.04*	1.17±0.11*	3.14±0.13*	14.29±1.46*	1.53±0.09	*Lea* > *Cap* > *Bol* / *Tat*	*Lea* > *Cap* > *Bol* / *Tat*
Root	1	1.06±0.10	2.84±0.49	1.21±0.18	0.40±0.05	3.06±0.36	12.11±1.76	7.06±0.79	2.52±0.23	*Cap* > *Lea* / *Bol* > *Tat*	*Cap* > *Lea* > *Bol* / *Tat*
	3	1.29±0.13	1.94±0.19	1.36±0.16	0.67±0.05	2.10±0.22	8.06±1.39	5.15±1.29	2.14±0.14	*Cap* > *Lea* / *Bol* > *Tat*	*Cap* / *Lea* > *Bol* / *Tat*
	6	0.57±0.08*	0.73±0.13*	1.52±0.16	0.52±0.04	1.60±0.20	4.13±0.52*	6.02±2.33	1.95±0.15	*Lea* > *Cap* / *Bol* / *Tat*	*Lea* > *Cap* / *Bol* / *Tat*

(b)		2 µM Cs in solution				8 µM Cs in solution				Tukey's between populations	
Plant fraction	K (mM)	*Bol*	*Cap*	*Lea*	*Tat*	*Bol*	*Cap*	*Lea*	*Tat*	2 µM Cs in solution	8 µM Cs in solution
Shoot	1	0.45±0.03	0.46±0.02	0.61±0.03	0.48±0.02	1.70±0.06	1.46±0.08	2.11±0.08	1.55±0.12	*Lea* / *Tat* / *Cap* / *Bol*	*Lea* / *Bol* / *Tat* / *Cap*
	3	0.45±0.02	0.48±0.01	0.52±0.04	0.44±0.02	1.73±0.03	1.64±0.08	2.00±0.13	1.68±0.07	*Lea* / *Cap* / *Bol* / *Tat*	*Lea* / *Bol* / *Tat* / *Cap*
	6	0.37±0.03*	0.34±0.01*	0.40±0.01*	0.38±0.04	1.41±0.09	1.12±0.09*	1.80±0.10	1.61±0.09	*Lea* / *Tat* / *Bol* / *Cap*	*Lea* / *Tat* / *Bol* / *Cap*
Root	1	0.31±0.01	0.14±0.002	0.32±0.01	0.17±0.01	1.12±0.11	0.33±0.05	0.58±0.09	0.87±0.06	*Lea* / *Bol* > *Tat* / *Cap*	*Bol* / *Tat* > *Lea* > *Cap*
	3	0.26±0.02	0.04±0.01*	0.39±0.07	0.16±0.01	1.14±0.06	0.28±0.01	0.63±0.08	0.98±0.06	*Lea* > *Bol* / *Tat* > *Cap*	*Bol* / *Tat* > *Lea* > *Cap*
	6	0.24±0.01	0.05±0.01*	0.14±0.003*	0.13±0.02	0.72±0.05*	0.32±0.04	0.75±0.49	0.91±0.05	*Bol* > *Lea* / *Tat* > *Cap*	*Tat* / *Bol* > *Lea* > *Cap*

[*] $p<0.05$ for K levels for each population; [(†)] The populations are ordered from the highest to the lowest accumulation and interconnected by "/" in the case they are ns, or by ">" in the case of $p<0.05$

4.5. Discussion

Our results unambiguously confirm the postulated Tl hyperaccumulator status of the calamine *Biscutella laevigata* population from Les Avinières (*Lea*) (Anderson *et al.*, 1999). It showed all the characteristics of a true hyperaccumulator, these are, 1) an extremely high level of Tl accumulation in its foliage, even at relatively low levels of Tl exposure, 2) a shoot to root Tl concentration ratio higher than unity, 3) an extraordinary level of Tl tolerance. Furthermore, it has been shown to hyperaccumulate both in its natural environment but also, as shown in the present study, in greenhouse or climate room experiments that use either soil or hydroponics.

Tl hyperaccumulation is obviously not a species-wide property in *B. laevigata*. The non-metallicolous population from the Tatra Mountains (*Tat*) did not show any of the typical hyperaccumulator traits (see above). In agreement with field observations (Wierzbicka *et al.*, 2004), also the metallicolous population from Bolesław (*Bol*) appeared to lack any propensity for Tl hyperaccumulation. The calamine population from Cave del Predil (*Cap*) presents a special case. In their own natural environment, the Cave del Predil mine tailing, these plants often exceed the threshold criterion for foliar hyperaccumulation of Tl (Pošćić *et al.*, unpublished), which has been set at 1000 mg kg^{-1} dry weight (Krämer, 2010). In fact, according to this criterion, *Cap* plants hyperaccumulated also in our experiments, both in their native soil and in hydroponics, although they never displayed a hyperaccumulator-like Tl root-to-shoot translocation. However, as suggested by our hydroponics experiments, foliar Tl hyperaccumulation in the *Cap* population seems to occur exclusively at extremely high, normally lethal exposure levels. Therefore, it seems that the foliar Tl hyperaccumulation of the plants in their natural environment can be explained by their extreme Tl tolerance, in combination with the extraordinarily high soil Tl concentration at the Cave del Predil mine tailing. In other words, in the particular case of *Cap*, foliar Tl hyperaccumulation may not be associated with specific physiological attributes other than (extreme) hypertolerance. To test this hypothesis, it should be checked in future experiments whether *Cap* plants are able to hyperaccumulate Tl also from less Tl-enriched soils, such as those from the Bolesław and Les Avinières mine tailings.

To date there is not much information on Tl tolerance in metallophytes. In the present study we consistently found significantly enhanced Tl tolerance (hypertolerance) in three metallicolous *B. laevigata* populations, in comparison with a non-metallicolous one. This

suggests that Tl is often present at potentially phytoxic concentrations in calamine soils. However, there was a huge variation in the degree of Tl hypertolerance among the metallicolous populations. It is not yet clear whether this variation is correlated with the degrees of soil Tl toxicity, but at least the most tolerant population (*Cap*) originates from the most Tl-enriched soil. On the other hand, *Lea* is much more Tl-tolerant than *Bol*, in spite of the more or less similar degrees of Tl-enrichment at their sites of origin. Therefore, it may be speculated that the high level of tolerance in *Lea* may have something to do with the fact that the *Lea* population is the only unambiguous Tl hyperaccumulator.

Regarding Cs accumulation and tolerance, our study clearly shows that there is little or no correspondence with the patterns observed for Tl. Cs tolerance was just slightly higher in *Lea* and *Cap* than in *Bol* and *Tat*, in sharp contrast with the huge difference in Tl tolerance. Moreover, there was no significant inter-population variation in Cs accumulation in root and shoot, except for *Cap*, which retained less Cs in its roots than any of the other populations. Even the Tl hyperaccumulating population (*Lea*) did not show any increased foliar accumulation of Cs, in comparison with the other populations. In addition, the patterns of K-imposed inhibition of Cs accumulation were generally less pronounced and very different from those found for Tl accumulation. Therefore, it can be concluded safely that Cs and Tl accumulation use different pathways, although both are K analogues. From the phytoremediation viewpoint it may be interesting that there was a very low degree of Cs retention in roots in the *Cap* population, but this was, unfortunately, not associated with a higher accumulation in the shoot. However, we only screened four populations, and the possibility remains that variation in shoot Cs accumulation does exist. In any case, Cs accumulation capacity is not predictable from Tl accumulation.

Acknowledgements

We thank Alicja A. Kostecka (Władysław Szafer Institute of Botany, Polish Academy of Sciences, Poland) for providing Polish *Biscutella laevigata* seeds. Technical assistance by Ahmad Mohtadi (University of Isfahan, Iran) and Mazhar Iqbal (Vrije Universiteit Amsterdam, The Netherlands) are gratefully acknowledged.

5
General discussion

Filip Pošćić

5.1. Ecological characterization of *Biscutella laevigata* subsp. *laevigata*

An ecological survey on metallophytes in the former Pb-Zn Raibl mining site (Julian Alps, Italy) is described in chapter 2. Specimens of *Alyssum wulfenianum*, *Biscutella laevigata* subsp. *laevigata*, *Minuartia verna* and *Thlaspi rotundifolium* subsp. *cepaeifolium* were collected from metal polluted soils and from nearby natural native soil which had not been disturbed by any mining activity. As expected, Cd, Pb, Tl and Zn concentrations in metal polluted soils were anomalous, while the total concentrations of Cd, Pb and Zn in native soil were not very high. However, a high Tl concentration (average of 161 mg kg^{-1}) was found in the soil samples collected in native soil. Results of interest are the observation of Tl hyperaccumulation in shoots in *B. laevigata* subsp. *laevigata* (the highest ever recorded, 32,661 mg kg^{-1}), *A. wulfenianum* and *M. verna*. The work in chapter 2 had shown that a possible selective pressure for Tl tolerance occurred in plants from Pb-Zn Raibl mining site. In fact, since a plant's metal tolerance is the result of a genotype × environment interaction (Pollard *et al.*, 2002), hypothetically, when plants colonize metalliferous soils there should be a strong selection favoring the metal-tolerant genotypes, causing a rapid increase in the average tolerance of the local populations and leading to the establishment of metal-tolerant ecotypes. In order to confirm this hypothesis, it was necessary to test the Tl tolerance and population genetic structure through more populations which has been done in chapters 3 and 4.

5.2. Population genetics of *Biscutella laevigata* subsp. *laevigata*

The population genetic structure analysis is useful for disentangling selective and demographic history (chapter 3). Populations were compared for Tl accumulation in field conditions at a regional scale (NE Italy) and population genetic structure was investigated using Amplified Fragment Length Polymorphism (AFLP) markers. There were included both metallicolous and non-metallicolous populations. Tl hyperaccumulation in shoots was observed only in specimens from the former Pb-Zn Raibl mining site as mentioned before. On the other hand, genetic variability of metallicolous and non-metallicolous populations was not significantly different and the majority of the genetic variation (67.6%) was within population which is in agreement with those obtained for other metallophyte species (Verkleij *et al.*, 1989; Vekemans *et al.*, 1992; Mengoni *et al.*, 2000; Baumbach and Hellwig, 2003, 2007; Basic *et al.*, 2006). However the population from the former Pb-Zn Raibl mining site was clearly differentiated in cluster and ordination

analysis. The answer to the question, whether the high Tl contamination levels at the former Pb-Zn Raibl mining site have been sufficient to provoke the evolution of Tl hypertolerance in the local *B. laevigata* subsp. *laevigata* population was provided by experiments in chapters 3 and 4.

5.3. Caesium and thallium tolerance

Caesium and thallium tolerance in *B. laevigata* is described in chapter 4. Indications given in chapters 2 and 3, that a local population of *B. laevigata* subsp. *laevigata*, from the former Pb-Zn Raibl mining site, evolved a Tl hypertolerance are confirmed in chapter 4. To elucidate population differentiation for Tl tolerance, four *Biscutella laevigata* populations were tested for Tl tolerance and accumulation (two from mine sites, one from a waste heap and one from an uncontaminated soil). Since Cs has chemical similarity to Tl, we supposed a possible shared mechanism of uptake and we included also this element in our analysis. For this research purpose, seedlings were grown hydroponically and exposed to a series of Cs or Tl increasing concentrations. The results show unambiguously that a true Tl hyperaccumulation in *B. laevigata* subsp. *laevigata* is only present in the one from former Pb-Zn Les Avinières mining site in France while the population from the former Pb-Zn Raibl mining site consists of hypertolerant plants with no active translocation from roots to shoots. It is interesting that the higher capacity of accumulation in the France population was also confirmed after these plants have been growing in the original polluted soil from the former Pb-Zn Raibl mining site. Thus clearly indicating that a particular population adaptation evolved in plants form former Pb-Zn Les Avinières mining site. However, it is likely that the metal-polluted environment and the metalliferous native soils exert a selective pressure in the local population of *B. laevigata* subsp. *laevigata* in the former Pb-Zn Raibl mining site, in the form of Tl hypertolerance. Moreover, this population has shown a Cs hypertranslocation, demonstrated for the first time.

We also tested the four populations for Tl and Cs accumulation in their interaction with different K concentrations and we confirmed an involvement of Na/K pumps.

5.4. Conclusions and future perspectives

Our results are of some interest for application in Tl phytomining and Cs contaminated soil remediation. We offered insights into a possible involvement of Na/K pumps in

hypertolerance and hyperaccumulation of monovalent metals and population for future studies. Therefore, on one hand, we can plan management approach for Cs and Tl phytoremediation. One of the phytoremediation processes is the phytoextraction, which involves the removal of pollutants through the harvest of the aboveground biomass of tolerant (hyper)accumulators species. On the other hand the phytostabilization involves the establishment of a permanent green cover to immobilize the pollutants in the rhizosphere and to reduce the wind and water erosion.

A future experimental activity could consist in a field experiment for Tl remediation and a response to the radioactive ^{137}Cs isotope for its phytoremediation. Moreover in literature we can find similar results to ours that Tl and Cs are transported by K transporters. Therefore, variation in uptake or translocation is expected to be associated with altered expression/regulation or structural changes of particular K transporters like for CNGC1 (Kanter *et al.*, 2010). These, but also other types of candidate genes may be sampled from transcriptomics comparisons, through next generation sequences facilities, between *B. laevigata* contrasting phenotypes and *Arabidopsis thaliana*, since both are *Brassicaceae*. *B. laevigata* is a suitable species for this kind of analysis, as it presents a broad array of different locally adapted populations with specific high-level metal tolerances. In particular, analyses of the co-segregation patterns between specific metal hypertolerances and the expression of candidate Tl and Cs transporter in intra-specific crosses between plants from populations with contrasting tolerance phenotypes might provide clues regarding to the mechanisms involved. In alternative, since *B. laevigata* is probably not readily genetically accessible, we could check the transport/accumulation phenotypes of the corresponding *A. thaliana* mutants.

6
References

Adriano DC. 2001. Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risks of metals. 2nd ed. New York: Springer-Verlag.

Al-Attar AF, Martin MH, Nickless G. 1988. Uptake and toxicity of cadmium, mercury and thallium to *Lolium perenne* seedlings. Chemosphere 17:1219–1225.

Allus MA, Brereton RG, Nickless G. 1988. The effect of metals on the growth of plants: the use of experimental design and response surfaces in a study of the influence of Tl, Cd, Zn, Fe and Pb on barley seedlings. Chemometrics and Intelligent Laboratory Systems 3:215–231.

Al-Najar H, Kaschl A, Schulz R, Römheld V. 2005. Effect of thallium fractions in the soil and pollution origin on Tl uptake by hyperaccumulator plants: A key factor for assessment of phytoextraction. International Journal of Phytoremediation 7:55–67.

Anderson CWN, Brooks RR, Chiarucci A, LaCoste CJ, Leblanc M, Robinson BH, Simcock R, Stewart RB. 1999. Phytomining for nickel, thallium and gold. Journal of Geochemical Exploration 67:407−415.

Antonovics J, Bradshaw AD, Turner RG. 1971. Heavy metal tolerance in plants. Advances in Ecological Research 7:1−85.

Assunção AGL, Martins, PDaC, De Folter S, Vooijs R, Schat H, Aarts MGM. 2001. Elevated expression of metal transporter genes in three accessions of the metal hyperaccumulator *Thlaspi caerulescens*. Plant Cell and Environment 24:217–226.

ASTM. 1972. Standard test method for particle-size analysis of soils. D 422-63. Annual Book of ASTM Standards 04.08:117-127. American Society for Testing and Materials, Philadelphia.

Avery SV, Codd GA, Gadd GM. 1991. Caesium accumulation and interactions with other monovalent cations in the cyanobacterium *Synechocystis* PCC 6803. Journal of General Microbiology 137:405–413.

Baker AJM, Brooks RR. 1989. Terrestrial higher plants which hyperaccumulate metallic elements – a review of their distribution, ecology and phytochemistry. Biorecovery 1:81−126.

Baker AJM. 1981. Accumulators and excluders – strategies in the response of plants to heavy metals. Journal of Plant Nutrition 3:643–654.

Baker AJM. 1987. Metal tolerance. New Phytologist 106 (Suppl):93−111.

Baker AJM., Walker PL. 1990. Ecophysiology of metal uptake by tolerant plants. In: Heavy Metal Tolerance in Plants: Evolutionary Aspects. Shaw, A.J., Ed., CRC Press, Boca Raton, p 155–177.

Basic N, Salamin N, Keller C, Galland N, Besnard G. 2006. Cadmium hyperaccumulation and genetic differentiation of *Thlaspi caerulescens* populations. Biochemical Systematics and Ecology 34:667−677.

Batty LC. 2005. The potential importance of mine sites for biodiversity. Mine Water and the Environment 24:101−103.

Baumbach H, Hellwig FH. 2003. Genetic variation within and among metal-tolerant and non-tolerant populations of *Armeria maritima* (Mill.) Willd. s. l. in Central and Northeast Germany. Plant Biology 5:186–193.

Baumbach H, Hellwig FH. 2007. Genetic differentiation of metallicolous and non-metallicolous *Armeria maritima* (Mill.) Willd. taxa (*Plumbaginaceae*) in Central Europe. Plant Systematics and Evolution 269:245–258.

Becker T, Dierschke H. 2008. Vegetation response to high concentrations of heavy metals in the Harz Mountains, Germany. Phytocoenologia 38:255–265.

Bert V, MacNair MR, De Laguerie P, Saumitou-Laprade P, Petit D. 2000. Zinc tolerance and accumulation in metallicolous and non-metallicolous populations of *Arabidopsis halleri* (*Brassicaceae*). New Phytologist 146:225–233.

Bizoux J -P, Daïnou K, Raspé O, Lutts S, Mahy G. 2008. Fitness and genetic variation of *Viola calaminaria*, an endemic metallophyte: implications of population structure and history. Plant Biology 10:684–693.

Bonin A, Bellemain E, Eidesen PB, Pompanon F, Brochmann C, Taberlet P. 2004. How to track and assess genotyping errors in population genetics studies. Molecular Ecology 13:3261−3273.

Bonin A, Ehrich D, Manel S. 2007. Statistical analysis of amplified fragment length polymorphism data: a toolbox for molecular ecologists and evolutionists. Molecular Ecology 16:3737−3758.

Boyd RS, Martens SN. 1992. The raison d'être for metal hyperaccumulation by plants. In: The Vegetation of Ultramafic (Serpentine) Soils. Baker AJM, Proctor J, and Reeves RD, Eds., Intercept Ltd., Andover, p 279–289.

Boyd RS, Martens SN. 1998. Nickel hyperaccumulation by *Thlaspi montanum* var. *montanum* (*Brassicaceae*): a constitutive trait. American Journal of Botany 85:259–265.

Bradshaw AD, McNeilly T. 1981. Evolution and Pollution. London: Edward Arnold.

Branquinho CH, Serrano C, Pinto MJ, Martins-Loução MA. 2007. Revisiting the plant hyperaccumulation criteria to rare plants and earth abundant elements. Environmental Pollution 146:437−443.

Brown G. 2001. The heavy-metal vegetation of north-western mainland Europe. Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie 123:63−110.

Burt RO. 1993. Cesium and cesium compounds, in Kirk-Othmer encyclopedia of chemical technology (4th ed.): New York, John Wiley and Sons, Inc., v 5, p 749–764.

Butterman WC, Brooks WE, Reese RG Jr (2005) Cesium. U.S. Geological Survey, Reston, Virginia

Bystrzejewska-Piotrowska G, Urban PŁ. 2003. Accumulation of cesium in leaves of *Lepidium sativum* and its influence on photosynthesis and transpiration. Acta Biologica Cracoviensia Series Botanica 45:131−137.

Cannon HL. 1960. Botanical prospecting for ore deposit. Science 132:591−598.

Cataldo DA, Wildung RE. 1978. Soil and plant factors influencing the accumulation of heavy metals by plants. Environmental Health Perspectives 27:149–159.

Commission of the European Communities. 1991. CORINE-biotopes manual. Habitats of the European Community. A method to identify and describe consistently sites of major importance for nature conservation. EUR 12587/3.

Conover WJ, Iman RL. 1982. Analysis of covariance using the rank transformation. Biometrics 38:715–724.

Conti F, Abbate G, Alessandrini A, Blasi C. 2005. An annotated Checklist of the Italian vascular flora. Roma: Palombi.

Davis JJ. 1963. Cesium and its relationships to potassium in ecology. In: Radioecology, Schultz V. and Klement AW, eds, Reinhold, New York, p 539–556.

Di Colbertaldo D. 1948. Il Giacimento Piombo Zincifero di Raibl in Friuli (Italia). Roma: Pubbl. S.A. Miniere Cave di Predil (Raibl).

Dice LR. 1945. Measures of the amount of ecologic association between species. Ecology 26:297–302.

Doyle JJ, Doyle JL. 1990. Isolation of plant DNA from fresh tissue. Focus 12:13−15.

Dushenkov S. 2003. Trends in phytoremediation of radionuclides. Plant and Soil 249:167–175.

Ernst WHO, Knolle F, Kratz S, Schnung E. 2004. Aspects of toxicology of heavy

metals in the Harz region – a guided excursion. Landbauforschunf Völkenrode 54:53–71.

Ernst WHO, Verkleij JAC, Schat H. 1992. Metal tolerance in plants. Acta Botanica Neerlandica 41:229–248.

Ernst WHO. 1974. Schwermetallvegetation der Erde. Stuttgart: Gustav Fischer Verlag.

Escarré J, Lefèbvre C, Raboyeau S, Dossantos A, Gruber W, Cleyet Marel JC, Frérot H, Noret N, Mahieu S, Collin C, van Oort F. 2011. Heavy metal concentration survey in soils and plants of the Les Malines mining district (Southern France): implications for soil restoration. Water Air and Soil Pollution 216:485−504.

Excoffier L, Lischer HEL. 2010. Arlequin suite ver 3.5: A new series of programs to perform population genetics analyses under Linux and Windows. Molecular Ecology Resources 10:564–567.

Excoffier L, Smouse PE, Quattro JM. 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131:479–491.

Faucon M-P, Ngoy Shutcha M, Meerts M. 2007. Revisiting copper and cobalt concentrations in supposed hyperaccumulators from SC Africa: influence of washing and metal concentrations in soil. Plant Soil 301:29–36.

Felsenstein J. 2005. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle.

Fischer M, Oswald K, Adler W. 2008. Exkursionsflora für Österreich, Liechtenstein und Südtirol. Freistadt: Werkstatt Plöchl Druck.

Gadd GM. 1992. Metals and microorganisms: a problem of definition. FEMS Microbiol. Lett. 100:197–204.

Galván-Arzate S, Santamaría A. 1998. Thallium toxicity. Toxicology Letters 99:1–13.

Ghosh, A, Sharma A, Talukder G. 1993. Effects of cesiumon cellular systems. Biological Trace Element Research 38:165–203.

Greenwood NN, Earnshaw A. 1984. Chemistry of the Elements. Pergamon Press, Oxford.

Günther K, Umland F. 1989. Bonding states of thallium and cadmium in thallium-treated and native rape. Journal of Inorganic Biochemistry 36:63–74.

Hampton CR, Bowen HC, Broadley MR, Hammond JP, Mead A, Payne KA, Pritchard J, White PJ. 2004. Cesium Toxicity in *Arabidopsis*. Plant Physiology 136:3824–3837.

Heim M, Wappelhorst O, Markert B. 2002. Thallium in terrestrial environments – occurrence and effects. Ecotoxicology 11:369–377.

Jalas J, Suominen J, Lampinen R, Kurtto A. 1999. Atlas Florae Europaeae: Distribution of Vascular Plants in Europe. 12. Resedaceae to Platanaceae. Committee for Mapping the Flora of Europe and Societas Biologica Fennica Vanamo, Helsinki.

Kabata-Pendias A. 2001. Trace Elements in Soil and Plants. Florida: CRC Press, Florida.

Kanter U, Hauser A, Michalke B, Dräxl S, Schäffner AR. 2010. Caesium and strontium accumulation in shoots of *Arabidopsis thaliana*: genetic and physiological aspects. Journal of Experimental Botany doi:10.1093/jxb/erq213.

Keith LH, Telliard WA. 1979. Priority pollutants: I. A perspective view. Environmental Science and Technology 13:416–423.

Komarov E, Bennett BG. 1983. Selected Radionuclides. World Health Organization, Geneva.

Krämer U. 2010. Metal hyperaccumulation in plants. Annual Review of Plant Biology 61:517−534.

Krasnodębska-Ostręga B, Asztemborska M, Golimowski J, Strusińska K. 2008. Determination of thallium forms in plant extracts by anion exchange chromatography with inductively coupled plasma mass spectrometry detection (IC-ICP-MS). Journal of Analytical Atomic Spectrometry 23:1632–1635.

LaCoste C, Robinson B, Brooks RR, Anderson C, Chiarucci A, Leblanc M. 1999. The phytoremediation potential of thallium-contaminated soils using *Iberis* and *Biscutella* species. International Journal of Phytoremediation 4:327−338.

Lambinon J, Auquier P. 1963. La flore et la végétation des terrains calaminaires de la Wallonie septentrionale et de la Rhénanie aixoise. Types chorologiques at groupes écologiques. Nature mosana 16:113−131.

Leblanc M, Petit D, Deram A, Robinson BH, Brooks RR. 1999. The phytomining and environmental significance of hyperaccumulation of thallium by *Iberis intermedia* from Southern France. Economic Geology 94:109−114.

Legislative Decree no. 152 of 3 April 2006. Rules in environmental field. Italian Official Journal vol. 88 of 14 April 2006 – Ordinary supplement no. 96:275–368.

Ma LQ, Komar KM, Tu C, Zhang WH, Cai Y, Kennelley ED. 2001. A fern that hyperaccumulates arsenic − a hardy, versatile, fast growing plant helps to remove arsenic from contaminated soils. Nature 409:579.

Macnair MR, Baker AJM. 1994. Metal tolerance in plants: evolutionary aspects. In: Farago ME, ed. Plants and the chemnical elements. Weinheim, Germany: VCH, p 68–86.

Macnair MR, Bert V, Huitson SB, Saumitou-Laprade P, Petit D. 1999. Zinc tolerance and hyperaccumulation are genetically independent characters. Proceedings of the Royal Society B-Biological Sciences 266:2175−2179.

Macnair MR, Tilstone GH, Smith SE. 2000. The genetics of metal tolerance and accumulation in higher plants. In: Terry N, Bañuelos G, eds. Phytoremediation of Contaminated Soil and Water. Boca Raton: Lewis Publisher, p 235−250.

Macnair MR. 1993. The genetics of metal tolerance in vascular plants. New Phytologist 124:541−559.

Madejón P, Murillo JM, Marañón T, Lepp NW. 2007. Factors affecting accumulation of thallium and other trace elements in two wild *Brassicaceae* spontaneously growing on soils contaminated by tailings dam waste. Chemosphere 67:20–28.

Mantel N. 1967. The detection of disease clustering and a generalized regression approach. Cancer Research 27:209−220.

Manton I. 1937. The problem of *Biscutella laevigata* L. II. The evidence from meiosis. Annals of Botany (London) 5:439−465.

Marmo E, Matera MG, Acampora R, Vacca C, De Santis D, Maione S, Susanna V, Chieppa S, Guarino V, Servodio R, Cuparencu B, Rossi F. 1987. Prenatal and postanal metal exposure: effect on vasomotor reactivity development of pups: experimental research with antimony trichloride, thallium sulfate and sodium metavanadate. Current Therapeutic Research 42:823–838.

Martens DC, Lindsay WL. 1990. Testing soils for copper, iron, manganese, and zinc. In: Westerman RL, editor. Soil Testing and Plant Analysis. Soil Science Society of America, Madison, USA, p 229−264.

McNair M. 1987. Heavy metal tolerance in plants: A model evolutionary system.Trends in Ecology and Evolution 2:354−359.

Meerts P, Van Isacker N. 1997. Heavy metal tolerance and accumulation in metallicolous and nonmetallicolous populations of *Thlaspi caerulescens* from continental Europe. Plant Ecology 133:221–231.

Mengoni A, Gonelli C, Galardi F, Gabbrielli R, Bazzicalupo M. 2000. Genetic diversity and heavy metal tolerance in populations of *Silene paradoxa* L. (*Caryophyllaceae*): a random amplified polymorphic DNA analysis. Molecular Ecology 9:1319–1324.

Mengoni A, Schat H, Vangronsveld J. 2009. Plants as extreme environments? Ni-resistant bacteria and Ni-hyperaccumulators of serpentine flora. Plant Soil 331:5−16.

Moeschlin, S. 1980. Thallium poisoning. Clinical Toxicology 17:133–146.

Mougel C, Teyssier S, d'Angelo C, Groud K, Neyra M, Sidi-Boumedine K, Cloeckaert A, Peloille M, Baucheron S, Chaslus-Dancla E, Jarraud S, Meugnier H, Forey F, Vandenesch F, Lina G, Etienne J, Thioulouse J, Manceau C, Robbe P, Nalin R, Briolay J, Nesme X. 2001. Experimental and theoretical evaluation of typing methods based upon random amplification of genomic restriction fragments (AFLP) for bacterial population genetics. Genetics Selection Evolution 33:S319–S338.

Mulkey JP, Oehme FW. 1993. A review of thallium toxicity. Veterinary and Human Toxicology. 35:445–453.

Müller HW, Schwaighofer B, Kalman W. 1994. Heavy metal contents in river sediments. Water Soil Air Poll 72:191−203.

Nadal M, Schuhmacher M, Domingo JL. 2004. Metal pollution of soils and vegetation in an area with petrochemical industry. Science of the Total Environment 321:59−69.

Nei M, Li WH. 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proceedings of the National Academy of Sciences, USA 76:5269–5273.

Nelson DW, Sommers LE. 1996. Total Carbon, Organic Carbon, and Organic Matter. In: Sparks DL, editor. Methods of Soil Analysis, Part 3. Chemical Methods. Soil Science Soceity of America Book Series Number 5. American Society of Agronomy, Madison, WI, p 961−1010.

Nolan A, Schaumlöffel D, Lombi E, Ouerdane L, Lobinski R, McLaughlin M. 2004. Determination of Tl(I) and Tl(III) by IC-ICP-MS and application to Tl speciation analysis in the Tl hyperaccumulator plant *Iberis intermedia*. Journal of Analytical Atomic Spectrometry 19:757–761.

Olowokudejo JD, Heywood VH. 1984. Cytotaxonomy and breeding system on the genus *Biscutella* (*Cruciferae*). Plant Systematics and Evolution 145:291−309.

Ouborg NJ, Vriezen WH. 2007. An ecologist's guide to ecogenomics. Journal of Ecology 95:8−16.

Parisod C, Besnard G. 2007. Glacial in situ survival in the Western Alps and polytopic autopolyploidy in *Biscutella laevigata* L. (*Brassicaceae*). Molecular Ecology 16:2755−2767.

Parisod C, Christin P-A. 2008. Genome-wide association to fine-scale ecological heterogeneity within a continuous population of *Biscutella laevigata* (*Brassicaceae*). New Phytologist 178:436−447.

Parisod C. 2008. Postglacial recolonisation of plants in the western Alps of Switzerland. Botanica Helvetica 118:1−12.

Pavlíčková J, Zbíral J, Smatanová M, Habarta P, Houserová P, Kubáň V. 2006. Uptake of thallium from artificially contaminated soils by kale (*Brassica oleracea* L. var. *acephala*). Plant Soil and Environment 52:544–549.

Percival TJ, Radtke AS. 1994. Sedimentary-rock-hosted disseminated gold mineralization in the Alšar district, Macedonia. The Canadian Mineralogist 32: 649–665.

Peter ALJ, Viraraghavan T. 2005. Thallium: a review of public health and environmental concerns. Environment International 31:493−501.

Pollard AJ, Powell KD, Harper FA, Smith JAC. 2002. The genetic basis of metal hyperaccumulation in plants. Critical Reviews in Plant Sciences 21:539−566.

Pollard AJ. 2000. Metal hyperaccumulation: a model system for coevolutionary studies. New Phytologist 146:179−181.

Prick JJG. 1979. Thallium poisioning. In: Vinkrn, P.J., Bruyn, G.W. (Eds.),

Intoxication of the Nervous System, Handbook of Clinical Neurology, North-Holland, New York, v 36, p 239–278.

Punz W, Mucina L. 1997. Vegetation on anthropogenic metalliferous soils in the Eastern Alps.Folia Geobotanica and Phytotaxonomica 32:283−295.

Radić S, Cvjetko P, Glavas K, Roje V, Pevalek-Kozlina B, Pavlica M. 2009. Oxidative stress and DNA damage in broad bean (*Vicia faba* L.) seedlings induced by thallium. Environmental Toxicology and Chemistry 28:189–196.

Raffaelli M, Baldoin L. 1997. Il complesso di *Biscutella laevigata* L. (*Cruciferae*) in Italia. Webbia 52:87−128.

Rajakaruna N. 2004. The edaphic factor in the origin of plant species. International Geology Review 46:471−478.

Ralph L, Twiss MR. 2002. Comparative toxicity of Tl(I), Tl(III) and Cd(II) to the unicellular alga *Chlorella* isolated from Lake Erie. Bulletin of Environmental Contamination and Toxicology 68:261–268.

Rascio N. 1977. Metal accumulation by some plants growing on Zinc-mine deposits. Oikos 29:250–253.

Reeves RD, Baker AJM. 1984. Studies on metal uptake by plants from serpentine and non-serpentine populations of *Thlaspi goesingense* Hálácsy (*Cruciferae*). New Phytologist 98:191−204.

Reeves RD, Baker AJM. 2000. Metal-accumulating plants. In: Raskin I, Ensley BD, editors. Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment. New York: Wiley, p 193−229.

Reeves RD, Brooks RR. 1983. Hyperaccumulation of lead and zinc by two metallophytes from mining areas of central Europe. Environmental Pollution Series A, Ecological and Biological 31:277−285.

Reeves RD. 2006. Hyperaccumulation of trace elements by plants. In: Morel JL, Echevarria G, Goncharova N, editors. Phytoremediation of Metal-Contaminated Soils. NATO Science Series: IV: Earth and Environmental Sciences, vol. 68. New York: Springer, p 25−52.

Richau KH, Schat H. 2009. Intraspecific variation of nickel and zinc accumulation and tolerance in the hyperaccumulator *Thlaspi caerulescens*. Plant and Soil 314:253−262.

Roosens NH, Willems G, Saumitou-Laprade P. 2008. Using *Arabidopsis* to explore zinc tolerance and hyperaccumulation. Trends in Plant Science 13:208−215.

Ross SM. 1994. Sources and forms of potentially toxic metals in soil-plant systems. In: Ross SM, ed. Toxic Metals in Soil – Plant Systems. Chichester, UK: Wiley, p 3−26.

Sager M. 1994. Thallium. Toxicological and Environmental Chemistry 45:11–32.

Saha A. 2005. Thallium toxicity: A growing concern. Indian Journal of Occupational and Environmental Medicine 9:53–56.

Saitou N, Nei M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution 4:406–425.

Schat H, ten Bookum WM. 1992. Genetic control of copper tolerance in *Silene vulgaris*. Heredity 68:219−229.

Schat H, Vooijs R, Kuiper E. 1996. Identical major gene loci for heavy metal tolerances that have evolved in different local populations and subspecies of *Silene vulgaris*. Evolution 50:1888–1895.

Schat H, Vooijs R. 1997. Multiple tolerance and co-tolerance to heavy metals in *Silene vulgaris*: a co-segregation analysis. New Phytologist 136:489–496.

Scheckel KG, Lombi E, Rock SA, McLaughlin MJ. 2004. In vivo synchrotron study of thallium speciation and compartmentation in *Iberis intermedia*. Environmental Science

and Technology 38:5095–5100.

Siegel BZ, Siegel SM. 1975. Selective role for potassium in the phytotoxicity of thallium. Bioinorganic chemistry 4:93–97.

Siegel BZ, Siegel SM. 1976a. Thallium antagonism toward potassium dependent systems. Bioinorganic chemistry 6:229–232.

Siegel BZ, Siegel SM. 1976b. Effect of potassium on thallium toxicity in cucumber seedlings: further evidence for potassium-thallium ion antagonism. Bioinorganic chemistry 6:341–345.

Smith RF. 1979. The occurrence and need for conservation of metallophytes on mine wastes in Europe. Environmental Geochemistry and Health 4:131–147.

Smith SE, Macnair MR. 1998. Hypostatic modifiers cause variation in the degree of copper tolerance in *Mimulus guttatus*. Heredity 80:760–768.

Soil Survey Division Staff. 1993. Soil survey manual. Soil Conservation Service. U. S. Department of Agriculture, Notebook 18.

Stevanović B, Dražić G, Tomović G, Šinžar-Sekulić J, Melovski Lj, Novović I, Marković DM. 2010. Accumulation of arsenic and heavy metals in some *Viola* species from an abandoned mine, Alchar, Republic of Macedonia (FYROM). Plant Biosystems 144:644–655.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution 28:2731–2739.

Teertstra DK, Cerny P, Chapman R. 1992. Compositional heterogeneity of pollucite from High Grade Dyke, Maskwa Lake, southeastern Manitoba. The Canadian Mineralogist 30:687–697.

Thioulouse J, Dray S. 2007. Interactive Multivariate Data Analysis in R with the ade4 and ade4TkGUI Packages. Journal of Statistical Software 22:1–14.

Tremel A, Masson P, Garraud H, Donard OFX, Baize D, Mench M. 1997. Thallium in French agrosystems – II. Concentration of thallium in field-grown rape and some other plant species. Environmental Pollution 97:161–168.

Tremetsberger K, König C, Samuel R, Pinsker W, Stuessy TF. 2002. Infraspecific genetic variation in *Biscutella laevigata* (*Brassicaceae*): new focus on Irene Manton's hypothesis. Plant Systematics and Evolution 233:163−181.

United States Department of Agriculture. 2000. Soil quality – Urban technical note No. 3

USEPA. 1995a. EPA Method 3051: Microwave assisted acid digestion of sediments, sludges, soils, and oils. In: Test Methods for Evaluating Solid Waste, 3rd ed. Washington, DC.

USEPA. 1995b. EPA Method 3052: Microwave assisted acid digestion of siliceous and organically based matrices. In: Test Methods for Evaluating Solid Waste, 3rd ed. Washington, DC.

Vekemans X, Lambert A, Lefebvre C. 1992. Isozyme variation at the populational level in *Armeria maritima*. Belgian Journal of Botany 125:270–275.

Verkleij JAC, Bast-Cramer WB, Koevoets P. 1989. Genetic studies in populations of *Silene cucubalus*occurring on various polluted and unpolluted areas. In: Scholz F, Gregorius HR, Rudin D, editors. Genetic effects of air pollutants in forest tree populations: Springer-Verlag Publishers, p 107–114.

Verkleij JAC, Lugtenborg TF, Ernst WHO. 1989. The effect of geographical isolation on enzyme polymorphism of heavy-metal tolerant populations of *Minuartia verna* (L) Hiern. Genetica 78:133–143.

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Paleman J, Kuiper M, Zabeau M. 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23:4407−4414.

Wenzel WW, Jockwer F. 1999. Accumulation of heavy metals in plants grown on mineralised soils of the Austrian Alps. Environmental Pollution 104:145−155.

White PJ, Broadley MR. 2000. Tansley Review No. 113. Mechanisms of caesium uptake by plants. New Phytologist 147:241–256.

Whiting SN, Reeves RD, Richards D, Johnson MS, Cooke JA, Malaisse F, Paton A, Smith JAC, Angle JS, Chaney RL, Ginocchio R, Jaffré T, Johns R, McIntyre T, Purvis OW, Salt DE, Schat H, Zhao FJ, Baker AJM. 2004. Research priorities for conservation of metallophyte biodiversity and their potential for restoration and site remediation. Restoration Ecology 12:1065−116.

Wierzbicka M, Pielichowska M. 2004. Adaptation of *Biscutella laevigata* L, a metal hyperaccumulator, to growth on a zinc – lead waste heap in southern Poland I: Differences between waste-heap and mountain populations. Chemosphere 54:1663–1674.

Wierzbicka M, Szarek-Łukaszewska G, Grodzińska K. 2004. Highly toxic thallium in plants from the vicinity of Olkusz (Poland). Ecotoxicology and Environmental Safety 59:84−88.

Wisskirchen R, Haupler H. 1998. Standardliste der Farn-und Blütenpflanzen Deutschlands. Stuttgart: Eugen Ulmer.

Xiaoa T, Guhab J, Boylec D, Liua C-Q, Chena J. 2004. Environmental concerns related to high thallium levels in soils and thallium uptake by plants in southwest Guizhou, China. The Science of the Total Environment 318:223–244.

Yanqun Z, Yuan L, Jianjun C, Haiyan C, Li Q, Schwartz C. 2005. Hyperaccumulation of Pb, Zn and Cd in herbaceous grown on lead-zinc mining area in Yunnan, China. Environment International 31:755−762.

Zabeau M. 1993. Selective restriction fragment amplification: a general method for DNA fingerprinting. European Patent Application No. 0-534-858-A1.

Zbíral J, Pavlíčkova J, Havlíková S, Čižmárová E, Němec P, Sáňka M, Kubáň V, Medek P. 2002. Comparison of several soil extractants for determination of thallium. Communications in Soil Science and Plant Analysis. 33:3303−3312.

Zhao FJ, Lombi E, McGrath SP. 2003. Assessing the potential for zinc and cadmium phytoremediation with the hyperaccumulator *Thlaspi caerulescens*. Plant Soil 249:37−43.

Zitko V. 1975. Toxicity and pollution potential of thallium. The Science of the Total Environment 4:185–192.

Zyka V.1972. Thallium in plants from Alšar. Sborník geologických věd. Technologie, geochemie 10:91–96.

7
Summary

In an ecological survey on metallophytes belonging to the *Thlaspietum cepaeifolii* plant community (former Pb-Zn Raibl mining site, Italy) Tl hyperaccumulation was strongly confirmed in the pseudometallophye *Biscutella laevigata* subsp. *laevigata* shoots. Moreover Tl hyperaccumulation in shoots was discovered in *Alyssum wulfenianum* and *Minuartia verna* (Fellet *et al.,* Metallophytes and thallium hyperaccumulation at the former Pb-Zn mining site in Cave del Predil, Italy).

To test the hypothesis that says that when pseudometallophytes colonize metalliferous soils a strong selection favouring the metal-tolerant genotypes occurs, we combined ecological and genetic approach to investigate intraspecific variation of metal accumulation in 15 populations of *B. laevigata* subsp. *laevigata* in North-East Italy. The data on metal concentrations and genetic analysis indicate that a differentiation of local population of *B. laevigata* subsp. *laevigata* has occurred in the former mining site of Cave del Predil. On the other hand, this was not the case for other two metallicolous populations who are distinguished from non-metallicolous population by ecological factors but not by genetics ones (Poscic *et al.,* Ecological and genetic differentiation at regional scale among metallicolous and non-metallicolous population of the pseudometallophyte *Biscutella laevigata* subsp. *laevigata*).

For studying deeply Tl accumulation and toxicity, deeper investigation on Tl tolerance was necessary comparing the Cave del Predil population with other Tl metallicolous populations. Therefore Tl and Cs tolerance, in different K concentration hydroponic cultures, where examined in one non-metallicolous from Tatra Mountains (*Tat*, P)and the three known Tl metallicolous populations of *B. laevigata*: Cave del Predil (*Cap*, I), Les Avinières (*Lea*, F) and Bolesław (*Bol*, P). Caesium was chosen because of chemical similarity to Tl and K. *Cap* and *Lea* exhibited extremely high Tl tolerance and it was demonstrated for the first time a true shoot Tl hyperaccumulation in *Lea* mine plants. On the other hand, *Tat* and *Bol* populations exhibit high Tl toxicity evidence. However, Cs was efficiently uptaken by all the populations and did not show almost any toxicity symptoms. It was notable the high Cs translocation capacity in *Cap* plants: up to three-thirteen times the level in roots. The remarkably high levels of Tl hypertolerance in *Cap*, hyperaccumulation in *Lea* and hypertranslocation of Cs in *Cap* might relate to evolutionary adaptation and probably to actively sequestration. Thallium and Cs absorption are combined to K channels, thus indicating involvement of Na/K pumps.